Protein p16 immunohistochemical expression in premalignant and malignant lesions in cervical histopathology specimens and its clinical impact

A Dissertation Submitted in Part Fulfillment of the rules and regulations for the M.D. Degree Branch III (Pathology) Examinations of The Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in April 2015

CERTIFICATE

This is to certify that this dissertation titled "***Protein p16 immunohistochemical expression in premalignant and malignant lesions in cervical histopathology specimens and its clinical impact***" is a bonafide work done by Dr Nivedita Suresh, in part fulfillment of rules and regulations for the M.D. Branch III (Pathology) Degree Examination of the Tamil Nadu Dr. M.G.R. Medical University, to be held in April 2015.

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ORIGINALITY REPORT

7%	2%	6%	0%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	"American Society of Cytopathology Abstracts: 53rd Annual Scientific Meeting", Cancer, 10/25/2005 Publication	<1%
2	Thomas, Asha, Umesh Mahantshetty, Sadhana Kannan, Kedar Deodhar, Shyam K. Shrivastava, Chandan Kumar-Sinha, and Rita Mulherkar. "Expression profiling of cervical cancers in Indian women at different stages to identify gene signatures during progression of the disease", Cancer Medicine, 2013. Publication	<1%
3	"Abstracts Presented for the Thirty-Seventh Annual Meeting of the Society of Gynecologic Oncologists", Gynecologic Oncology, 200604 Publication	<1%
4	"abstracts", International Journal of Gynecological Cancer, 9/2004 Publication	<1%
5	John C. Panos. "Usefulness of concurrent	<1%

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LIST OF ABBREVIATIONS

AAR	Age Adjusted Incidence Rate
CDK	Cyclin Dependent Kinase
CEA	Carcino Embryonic Antigen
CIN	Cervical Intraepithelial Neoplasia
CK	Cytokeratin
CTD	Carboxy Terminal Domain
DNA	Deoxyribo Nucleic Acid
ER	Estrogen Receptor
FHIT	Fragile Histidine Triad
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
IHC	Immuno Histo Chemistry
ISH	In Situ Hybridisation
MTS	Multiple Tumour Suppressor
NCR	Non Coding Region
NPV	Negative Predictive Value
ORF	Open Reading Frame

PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PPV	Positive Predictive Value
PR	Progesterone Receptor
RNA	Ribo Nucleic Acid
SCC	Squamous Cell Carcinoma
WHO	World Health Organisation

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ABSTRACT

TITLE OF THE ABSTRACT: Protein p16 immunohistochemical expression in premalignant and malignant lesions in cervical histopathology specimens and its clinical outcome.

DEPARTMENT : Pathology

NAME OF THE CANDIDATE : Nivedita Suresh

DEGREE AND SUBJECT : MD. Pathology

NAME OF THE GUIDE : DR. Ramani Manoj Kumar.

OBJECTIVES:

a. To evaluate the diagnostic utility of p16 to differentiate between the premalignant and malignant squamous and glandular lesions of cervix.
b. To assess the utility of p16 to distinguish between benign and dysplastic lesions of cervix.
c. To assess the clinical impact of p16 expression on the disease outcome.
d. To assess the performance of cytology as a screening tool compared to histology.

METHODS: 120 cervical biopsies (15 cases each of CIN 1, 2, 3 and squamous cell carcinoma, 22 cases of adenocarcinoma and 38 reactive cases) were studied and p16 immunohistochemistry were performed. Score out of 8 was given based on intensity and proportion of p16 expression. Corresponding cytological diagnoses were also evaluated.

RESULTS: Sensitivity of p16 in CIN 1, 2, 3, squamous cell carcinoma, adenocarcinoma were found to be 73.7%, 93.3%, 93.3%, 100% and 95.4% respectively. Specificity of p16

in cervical squamous and glandular lesions was 85.7 % and 80 % respectively. p16 over expression also correlated with stage of tumour, lymph node metastasis, local and distant metastasis. Intensity of expression correlated better with lesion severity than proportion. It did not correlate with grade of the tumour. 15.7 % of reactive epithelium showed patchy weak cytoplasmic positivity for p16.

Cytology had sensitivity of 35.7 %, 69.2% and 50% for low grade lesions, high grade lesions and glandular lesions respectively.

The time taken for a dysplastic lesion to progress into a higher grade lesion was 16 months. Among the morphological parameters used to diagnose cervical dysplasia, mitotic count was found to be the only parameter which showed statistically significant difference among the subtypes. Koilocytosis was significantly associated with CIN 1 and CIN 2 but not with CIN 3. All the cases of koilocytosis were negative for p16. The most common presenting symptom was vaginal discharge in CINs and cervical growth in carcinoma. 15.8 % of women with CIN 1 were referred for biopsy due to abnormality in Pap smear.

CONCLUSION: p16 is a reliable marker to detect cervical dysplastic lesions. It can differentiate benign mimics from dysplastic lesions. Over expression of p16 is associated with higher stage of tumour, nodal and distant metastasis. Pap smear test alone has a low sensitivity for low grade lesions.

Key words: p16, CIN, Squamous cell carcinoma, Adenocarcinoma, glandular lesions, Pap smear, cytology.

INTRODUCTION

Cervical cancer is the fourth most common cancer in the world following breast, colorectal and lung cancers (1). It accounted for 8 % of all cancers of women and 7 % of cancer related mortality in 2012(2). Cervical cancer has gained importance in the past few decades as being one of the few vaccine preventable cancers. Moreover, there is an effective screening tool (Pap smear testing) for early detection and management. The implementation of screening programmes and HPV vaccination strategy of all young women have lead to significant reduction in the incidence of cervical cancer in developed countries compared to less developed countries.

The cervical epithelium progresses through a series of dysplastic changes of increasing grades before the development of an invasive carcinoma. Papanicolaou stain was developed in 1928 and has been in use for the study of cervical exfoliative cytology since 1941. Routine Pap smear testing is advised in all women over 21 years of age and in 1988, Bethesda system of standardised reporting was developed. Various studies have aimed at assessing the sensitivity, specificity, positive and negative predictive values of Pap smear testing to determine its efficacy as a screening modality in diagnosing the premalignant and malignant lesions of cervix. It was found that Pap test has a variable sensitivity and reasonably high specificity, positive and negative predictive values.

Colposcopic examination and biopsy of cervix was the next advent in the spectrum of preventive measures for cervical carcinomas. There are characteristic histological changes associated with each grade of cervical intraepithelial neoplasia. However, there

was a high interobserver variability and low reproducibility in interpreting the cervical biopsies.

The identification of Human Papilloma Virus in 1976 as a causative agent in the development of cervical cancers was a landmark discovery in the history of cervical tumourigenesis. HPV DNA testing in the cervical scrape became the molecular method of screening for cervical cancer due to its high sensitivity. However, it was noted that HPV prevalence is high in women younger than 30 years of age and the mere presence of the virus does not translate into a neoplastic process. Hence, HPV testing as a routine screening technique was not recommended in young women due to its low specificity and high cost.

It was important to correctly identify the grade of cervical intraepithelial neoplasia (CIN) as the treatment and prognosis differ in various subtypes. It became essential to develop a marker for dysplasia with a high sensitivity, comparable to HPV DNA testing and high specificity comparable to cytology. When the molecular mechanism by which HPV causes cervical cancer was extensively studied, it was found that the viral oncoproteins E6 and E7 dysregulate the tumour suppressor proteins p53 and Rb. E7 after binding to Rb protein releases E2F-1 transcription factor, in turn causing elevated levels of p16, which is a cyclin dependent kinase inhibitor. Thus, it was logical to conclude that p16 would be an ideal candidate as biomarker to detect cervical dysplasia.

A number of studies have investigated the role of p16 in cytological and histopathological samples in various squamous and glandular lesions of cervix. Majority of the studies have confirmed the over expression of p16 in squamous and glandular dysplasia(3). It has also been proven that p16 can discriminate the benign mimics from preneoplastic and neoplastic conditions.

It was found that the concurrent use of p16 immunohistochemistry improved the interobserver agreement and reproducibility in the diagnosis of premalignant lesions of cervix (4). It was also believed that as p16 over expression occurs as a result of cell cycle dysregulation following HPV infection, it can be used to establish cervical origin in adenocarcinoma of the pelvic region (5).

It is widely known that even though HPV colonisation is highly prevalent in young women, 90 % of them clear within 2 years and only 10 % progress into neoplasia. Studies have been done to investigate the role of p16 in predicting the outcome of the premalignant lesion and to assess the metastatic potential of carcinomas(6).

Majority of studies which have tried to develop a panel of immunohistochemical markers for cervical carcinoma have included p16 in the primary panel. It is also suggested that p16 immunohistochemistry can be complemented by MIB-1 proliferation index and other newly recognised markers such as cyclin D1, ProExC, cyclin E and p21 to improve the diagnostic accuracy.

Thus, various studies have investigated the role of p16 immunohistochemical marker in cytological and histological samples to establish a diagnosis of cervical dysplasia by differentiating it from various benign mimics, to ascertain primary malignancy in cervix and to predict the outcome of the disease in terms of progression into a higher grade lesion and metastatic potential.

REVIEW OF LITERATURE

Epidemiology

Cervical cancer ranks fourth among all the other cancers in the world following breast, colorectal and lung cancers. According to the statistical information provided by the World Cancer Research International, 5,28,000 new cases of cervical cancer were diagnosed all over the world in 2012. Globally, it accounted for 8% of all cancers of women and 7% of cancer related deaths in that year. Developing countries faced the highest disease burden with an incidence of 4, 45,000 (84%) of the new cases diagnosed in a year of which about one fifth were diagnosed in India. The highest incidence rates were observed in Sub-Saharan Africa, Latin American and Caribbean countries and the lowest incidence rates were noted in North America and Oceania (1).

The highest age adjusted rate per 1, 00,000 population was in Malawi (75.9) followed by Mozambique (65.0) and Comoros (61.3). Squamous cell carcinoma accounted for 80% of cervical cancers, adenocarcinoma, about 15% and other carcinomas like adenosquamous, small cell and neuroendocrine together amounted to 5%. According to the data provided by SEER programme (Surveillance, Epidemiology and End Result) by National Cancer Institute, 0.7% of all cancer related deaths were due to cervical cancer. Five year survival rate (2004 to 2010) was found to be 67.9%. The incidence rates have been falling at an average rate of 1.2% per year and death rates by an average of 1.3% per year over the past 10 years (2002 to 2011) owing to effective surveillance and screening programmes. Life time risk of developing cervical cancer was found to be 0.7%(7).

GLOBOCAN 2012, an international agency for cancer research maintained by World Health Organisation (WHO) states that up to 70 % of global burden of cervical cancer

mortality falls on less developed countries and more than 20 % of new cervical cancer cases are diagnosed in India. A drastic difference in the incidence of cervical cancers was noticed between North America and sub Saharan Africa (6.6 vs. 34.8 per 1,00,000 women) and it was attributed to effective screening programmes enabling early detection and management(2).

In European cancer registry, the crude incidence rate was estimated to be around 10 new cases per 1, 00,000 females. The age of diagnosis ranged from 25 to 64 years. A drastic reduction in incidence was observed from 1988 onwards due to successful cervical screening programmes. 1 in 134 is estimated to be the lifetime risk of a woman to develop cervical cancer in UK. 66 % of the cases were squamous cell carcinoma and 15 % were adenocarcinoma(8).

In 2012, Dikshit et al studied the cancer mortality rate in India. The most common cause of cancer death in Indian women between 30 – 69 years of age was found to be cervical cancer (17.1%). It was also found that cervical cancer was less common in Muslim women than in Hindu women. The death rate due to cervical cancer was estimated to be 16 per 1, 00,000 women. Thus a 30 year old Indian woman has a 0.7 % risk of dying due to cervical cancer before the age of 70(9).

The National Cancer Registry Programme maintained by Indian Council of Medical Research states that Chennai urban population based registry shows the highest incidence of cervical carcinoma in India with an age adjusted incidence rate of 30.6 per 1, 00,000 women. However, district based registry showed the highest incidence in north eastern districts of Tamil Nadu (Age adjusted Incidence rate: 39.2 per 1, 00,000 women). This was noted in the following districts in descending order: Pondicherry, Villupuram, Cuddalore, Thiruvallur and Chennai. Among the urban population, Chennai

topped the list followed by Chandigarh (AAR: 26.9 / 1,00,000), Bhopal (22.2/ 1,00,000), Bangalore (19.8 / 1,00,00), Mumbai (15.4 / 1,00,000), Delhi (13.7 / 1,00,000) and Kolkata (13.0 / 1,00,000)(See Figure 1). Lowest incidence was noted in Jammu & Kashmir and north eastern states like Arunachal Pradesh and Manipur. Figure 2 shows comparison of incidence rates in various cities in India with that of other countries (10).

Figure 1: Comparison of age adjusted incidence rates between districts in India

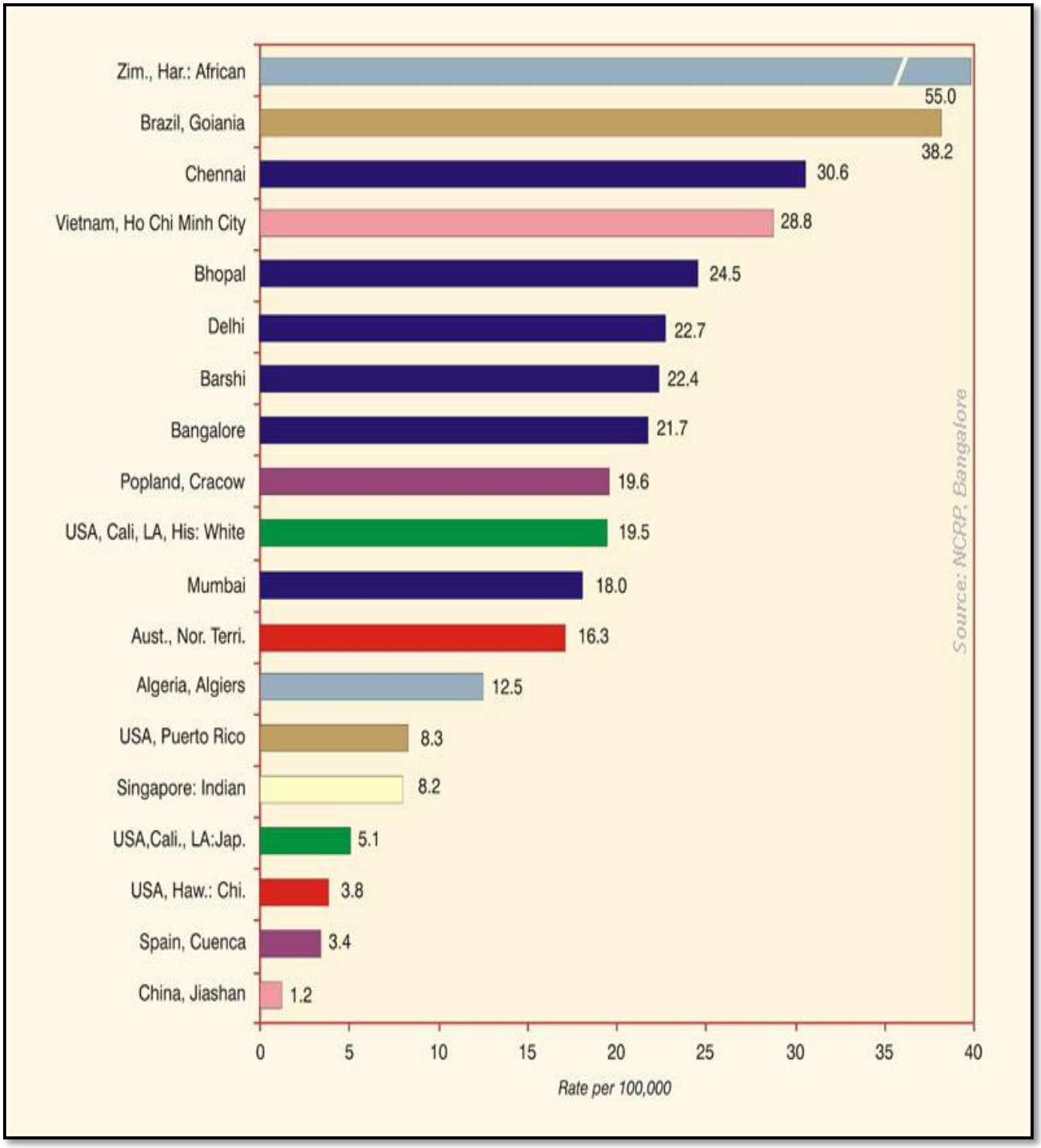

Figure 2: Comparison of age adjusted incidence rates of urban population based registry in India with that of other countries.

Normal anatomy, embryology and histology

The cervix is a fibromuscular cylindrical structure present in the lower portion of uterus, measuring about 2.5 to 3cm in length. A part of it is located above the vaginal vault whereas the remaining portion protrudes into the vagina. It develops from the two paramesonephric ducts by 6th week of gestation. By 12th week, the outer parts of the two ducts fuse to form a urogenital sinus which gives rise to cervix and uterus. The outer surface of the ectocervix is covered by stratified squamous epithelium, which continues into the vaginal mucosa. The endocervical canal consists of endocervical glands lined by mucin producing columnar epithelial cells, which merges with the stratified squamous epithelium of ectocervix in the lower end and continues into the lower uterine segment in the upper end, thus forming a bridge between vagina and endometrial cavity. This property of abrupt transition from one type of epithelium to another type makes the cervix vulnerable to dysplasia. Hence, a detailed understanding of the normal histology and progressive changes that occur to it is essential (11)(12).

Epithelium of the ectocervix: Sexually mature epithelium of ectocervix is non keratinised and is composed of mainly three layers of cells each with a characteristic morphology - the lower one third is formed by basal / parabasal cells, the middle one third by intermediate cells and the upper one third by superficial cells. The number of cells in each layer and the predominant cell type varies with age and the phase of menstrual cycle. Normal stratified squamous epithelium of the ectocervix demonstrates a differentiation sequence from the basal cells to superficial cells. In general, as the differentiation progresses the cells acquire more cytoplasm, the nuclei become smaller and the chromatin becomes paler(12)(13).

The basal layer is two to three cell layers thick and rests on a basement membrane. The cells are almost spherical, have an oval to round nucleus with an average size of 50 microns, which is perpendicular to the basal lamina with dense chromatin. Mitotic activity is usually inconspicuous. The parabasal cells are one to two layers thick, with nuclear size of approximately 40 to 45 microns and contain more cytoplasm compared to basal cells. These cells proliferate actively and hence mitotic figures are usually seen. The predominant function of these cells is regeneration of epithelium. Basal cells function as stem cells and parabasal cells form the replicating component. Hence these cells express epidermal growth factor receptors such as HER 2 neu, estrogen and progesterone receptors(11).

The middle one third is occupied by intermediate cells. They are polyhedral in shape, nuclear size measuring approximately 35 microns and exhibits gradual increase in volume of cytoplasm. The striking feature of these cells is that they begin to accumulate glycogen, thus rendering the cytoplasm clear and abundant, making them Periodic Acid Schiff positive and diastase labile. The nuclear size is stable up to the most superficial layer. These cells do not replicate. The significance of intermediate cells is that they can sometimes simulate koilocytic cells, leading to misinterpretation.

The top most layer is formed by superficial cells characterised by tiny pyknotic dot like nuclei, about 25 microns in size and contain abundant cytoplasm. This represents the most differentiated layer. Keratinisation can occur only in the superficial and intermediate cells.

In prepubertal and post menopausal women the squamous epithelium is atrophic due to lack of estrogen exposure. The predominant cell type seen is parabasal cell. There is no accumulation of intracytoplasmic glycogen. The significance of this is that it can be

confused with cervical intraepithelial neoplasia in cytology specimens. In sexually mature women, due to the presence of active form of estrogen, estradiol 17 beta, there is constant remodelling of the epithelium by proliferation, differentiation and exfoliation. A new population of cells is formed every 4-5 days. Progesterone inhibits maturation at the level of intermediate cells. Therefore, in the follicular phase of menstrual cycle, the predominant cell type seen on exfoliative cytology would be superficial cells, whereas in the luteal phase, it would be intermediate cells. During pregnancy, due to elevated levels of circulating progesterone, the intermediate cells predominate. The age and the phase of the menstrual cycle have to be borne in mind while interpreting exfoliative cytology to avoid a misdiagnosis(11)(13).

Mucin producing columnar epithelial cells line the surface as well as the glandular component in the endocervix. The columnar epithelial cells are tall with basally located nuclei, granular chromatin and vacuolated cytoplasm. The mucin stains for Periodic Acid Schiff and Alcian blue due to the presence of sulphated mucopolysaccharide. They are simple epithelia and express low molecular weight cytokeratins 7, 8, 18 and 19. Mitosis is usually absent. Ciliated cells are usually present. The cervical stroma is composed of fibrous connective tissue along with elastic fibres running parallel to smooth muscle bundles. An occasional lymphoid aggregate may be present. The sub-epithelial capillary network is well formed in the endocervix(11).

The cervix lies between the sterile environment of endometrial cavity and the microbial environment of vagina and hence has a strong immune apparatus. Other cells normally present in the cervix include Langerhans cells and cells of immune system as a part of host defence against bacterial and viral organisms. IgA antibody mediated, IgG antibody mediated and cellular immune systems are seen in the cervix. CD 8 positive T

lymphocytes are the predominant cells present followed by variable numbers of dendritic cells and B lymphocytes (13)(12).

Transformation zone: The squamocolumnar junction or the transformation zone is the point of transition between the stratified squamous epithelium of ectocervix and mucin producing columnar epithelium of endocervix. At birth, columnar epithelium lies in the ectocervix in majority of infants but it migrates into the endocervical canal soon after and remains there until menarche. However, with onset of puberty, the columnar epithelium moves again into the ectocervix, more towards the anterior region than the posterior. This is thought to occur due to swelling of cervical stroma under estrogenic stimulation which causes disproportionate increase in the bulk of uterus. This pulls the endocervical mucosa into the ectocervix and is referred to as ectropion. The exposed endocervical mucosa appears ulcerated and red to the naked eye. After this physiological ectropion, there is gradual replacement of columnar epithelium by stratified squamous epithelium during the reproductive years, thus forming the transformation zone which is the junction between the two different types of epithelia(12).

There are two types of squamocolumnar junction – the original transformation zone is the region where the native stratified squamous epithelium meets the endocervical columnar epithelium which is present in the ectocervix during reproductive years. After squamous metaplasia of columnar epithelium has occurred, the native squamocolumnar junction is the conversion point between the new and original squamous epithelium. The point where there is active replacement of columnar cells by squamous cells is the functional transformation zone. It often has an irregular contour and there is enlargement of the transformation zone during pregnancy and following estrogen

therapy. In short, in the early reproductive years, the functional transformation zone is near the anatomical external os and in the perimenopausal years, it lies deep in the endocervical canal away from the external os(12).

Two mechanisms have been proposed for migration of squamocolumnar junction – the direct ingrowth of native stratified squamous epithelium into the endocervix, pushing the columnar cells and dislocating them from the basement membrane which causes them to slough off. The second mechanism involves proliferation of reserve cells and their differentiation into squamous cells rather than columnar cells. The exact mechanism however is still not known(12).

The transformation zone is a region of active replication and replacement of endogenous epithelium thus making it highly susceptible to neoplastic transformation. The squamocolumnar junction can be seen with the aid of a colposcope, thus enabling the clinician to visualise structural changes that accompany neoplastic process, making it a valuable tool for early detection of intraepithelial neoplasia(14).

Knowledge of lymphatic supply of cervix is essential for sampling of lymph nodes in case of neoplasia. There are three major locations into which lymphatic drainage from cervix occurs – external iliac lymph nodes laterally, internal iliac lymph nodes posterolaterally and rectal and sacral nodes posteriorly. The descending branch of uterine artery forms the arterial supply and venous drainage is into the uterine vein. The pelvic splanchnic nerves (S2-S3) provide the nerve supply(12).

Morphological variants of cervical carcinoma:

Squamous cell carcinomas are usually exophytic masses that grow into the surface as polypoidal or papillary projections or can occasionally be endophytic lesions. They are broadly divided into two categories*: keratinising* and *non keratinising* depending on the evidence of keratin production. *Verrucous* carcinoma is a very well differentiated variant with an undulating and warty appearance. The neoplastic cells have abundant cytoplasm and features of HPV related changes are usually not conspicuous. *Warty or condylomatous* variant on the other hand, shows florid features of HPV infection like koilocytic change, multinucleation, papillomatosis, acanthosis, nuclear hyperchromasia and enlargement. *Papillary* variant shows broad and thin papillae of dysplastic cells extending into the stroma. They are usually associated with HPV 16. They lack cellular features of HPV and keratinisation*. Basaloid* squamous cell carcinoma is an aggressive variant and is usually associated with HPV 16. They are composed of nests of basaloid immature looking cells with focal evidence of keratinisation*. Lympho-epithelioma* like variant is composed of uniform small cells with vesicular nuclei, prominent nucleoli and scant cytoplasm in a background of lymphocytes. It differs from other variants in that the etiology is thought to be Epstein Barr Virus infection rather than HPV(15).

Adenocarcinoma can also present as exophytic masses in 50 % of the cases, others cause nodular enlargement and deep infiltration causing barrel shaped cervix. 15% do not cause visible lesions. Primary endocervical adenocarcinoma has to be distinguished from endometrial carcinomas by immunostaining for CEA and negative staining for ER, PR and vimentin. The commonest variant is *mucinous* adenocarcinoma, of which endocervical type accounts for about 70% of glandular neoplasms. Coexisting CIN can occur in up to 40 % of cases. They are usually well to moderately differentiated

tumours. Intestinal variant shows goblet cells and less frequently endocrine and Paneth cells. *Minimal deviation type* is a rare extremely well differentiated adenocarcinoma composed of glands lined by bland cells with occasional cells displaying moderate atypia and mitosis and are deeply invasive. It is difficult to make the diagnosis on punch biopsies. It is characterised by somatic mutation of STK 11, the gene that is responsible for Peutz Jeghers syndrome. The *villoglandular* variant shows frond like growth pattern and are usually well to moderately differentiated tumours. *Endometrioid* carcinomas are distinguished from uterine primary by less conspicuous squamous elements and little intracellular mucin. *Clear cell* carcinoma shows hob nailing of cells with solid, papillary or tubulocystic pattern (16).

Other less common variants: *Adenosquamous* carcinoma is composed of closely intermingled malignant squamous and glandular components. *Glassy cell* variant is a poorly differentiated tumour with a prevalence of 1-2 %. The neoplastic cells are large with distinct cell margins and ground glass cytoplasm. *Adenoid cystic* carcinoma of cervix show greater nuclear pleomorphism, necrosis and high mitotic rate compared to its counterpart in the salivary gland. *Mesonephric* variant is poorly differentiated and lack intracytoplasmic mucin and glycogen. *Serous* adenocarcinoma of cervix is identical to papillary serous carcinoma of ovary and endometrium and consists of complex papillary pattern with budding, atypical nuclei and psammomatous bodies. Another extremely rare variant is *hepatoid* adenocarcinoma (15).

Neuroendocrine tumours encompass a spectrum including carcinoid, atypical carcinoid, small cell and large cell carcinoma. They are differentiated based upon the mitotic activity and nuclear atypia. They are positive for neuroendocrine markers like neuron

specific enolase, synaptophysin and chromogranin. Undifferentiated carcinomas are the ones that do not show differentiation towards any specific lineage (14).

Viral oncogenesis and association of Human Papilloma Virus with cervical neoplasia.

The association of cervical neoplasia with Human Papilloma Virus is universally accepted. Various studies have proven that colonisation of oncogenic HPV DNA in the cervical epithelium precedes the development of cervical intraepithelial neoplasia. Human Papilloma Virus is made of a single molecule of circular double stranded DNA, measuring 8 kilo bases. To date, more than 100 HPV subtypes have been identified. Oncogenic HPV types are defined as "those which are associated with cervical or anal cancer and include HPV types 16, 18, 31, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82". The non oncogenic types, HPV 6, 11, 40, 42, 54 and 55 cause skin or mucosal warts. Of all the subtypes, HPV 16 is the commonest oncogenic strain with highest potential for persistence and malignant transformation of epithelial cells. HPV 16 is also the most frequently associated strain in case of squamous cell carcinoma whereas HPV 18 is the commonest strain detected in adenocarcinoma (11)(14).

Knowledge of HPV genome and gene protein interactions in host is essential to understand the role of p16 in cervical neoplasia. Almost all cervical cancers are caused by high risk HPV, most commonly the HPV 16 and 18 subtypes.

HPV genome: It consists of three coding regions required for viral replication and production of capsid protein envelope. The early (E) period of life cycle require 2 coding regions with six open reading frames (ORF) - E1, E2, E4, E5, E6, E7 which encode proteins required for transcription, replication, host cell transformation and integration

with host genome. The late (L) region encodes structural proteins (L1, L2). Non Coding Region (NCR) is involved in RNA synthesis and controls the expression of ORF(14).

E1 acts as DNA dependent ATPase and ATP dependent helicase. E2 is responsible for separation of HPV DNA into daughter and host cells. E6 and E7 are the proteins which impart oncogenicity to HPV. They are expressed in virtually all cervical neoplasias. They encode proteins which induce proliferation and transformation of keratinocytes. E6 protein binds to p53 and destroys it through ubiquitin dependent mechanism. p53 is a tumour suppressor gene that causes cell cycle arrest if DNA damage occurs. So the primary function of E6 is prevention of apoptosis. It also stimulates the expression of TERT which is the catalytic subunit of telomerase that removes the check in cell cycle thus causing immortalisation of cells. E7 binds to retinoblastoma protein causing dissociation of E2F-Rb complex and subsequent degradation of proteins. This triggers cell division by causing increased expression of enzymes required for nucleotide biosynthesis and cyclins that are required for progression through S phase in cell cycle. Thus, E7 mainly increases the rate of cell division. E7 and E6 proteins complement each other in their functions. E7 also causes inactivation of cyclin dependent kinase inhibitors p21 and p27 causing increased CDK4/cyclin D. Thus, the presence of E7 is implicated in the development of benign tumours and the presence of E6 in the development of malignant neoplasm(11).

HPV Life Cycle:

The transformation zone of cervix is the region where HPV infection begins and most of the neoplastic processes occur. In this region, HPV enters the basal or reserve cells through minor defects. The prevalence of HPV infections peaks between 20 to 24 years of age while there is a subsequent decline in prevalence due to development of

immunity against the virus. 90% of HPV infections are cleared within 2 years by the host immune response. The remaining proceeds to become (1) productive infection (2) abortive infections or (3) latent infections. In the productive infection, there is replication of the virus which kills the cells. In latent infections, there is persistence of virus in cytologically normal cells. The basal layer harbours HPV in the form of episomes (extra chromosomal DNA). Complete viral replication occurs only in epithelium undergoing terminal differentiation. Koilocyte formation can occur only in cells which are at least partially mature. Abortive infections occur when the virus in unable to complete its life cycle with integration of viral DNA into the host genome. This is thought to be the mechanism behind progression of CIN 3 to invasive carcinoma. Affinity of E7-Rb binding is considerably lower in low risk HPV and E6 protein may not bind to p53. Hence, the cell cycle dysregulation may occur by interfering with Notch signalling pathway(14).

In spite of the high incidence of HPV in sexually active women, there is a disproportionately low prevalence of cervical carcinoma. Therefore, it was concluded that a number of environmental factors also play an important role for transformation of HPV infected cells into a neoplastic process. The most important factors whose causal association have been proven are age of first sexual intercourse, number of partners, smoking, presence of sexually transmitted infections and immune status of the host.

A study done by Donna Dehn et al in 2007 investigated the different HPV detecting methods and relevance of molecular markers. The methods used were Direct Hybridisation, Hybrid capture 2, type specific PCR amplification, third wave invader genotype assay, viral load real time PCR and p16 immunohistochemistry. Hybrid capture 2 was found to be the best method with a sensitivity of more than 96% and

specificity of 61% in CIN 2 lesions. p16 immunohistochemistry had a sensitivity of 81.1% and specificity of 95.4 % for CIN 2 lesions. The authors proposed the use of p16 as a surrogate marker for detecting dysplastic lesions of cervix. ProExC was an emerging new method that included TOP2A and MCM2 molecular markers for detection of cervical dysplasia(17).

Another study done by Zhang Sheng et al in 2013, aimed at detection of subtypes of HPV in cervical glandular lesions using In situ Hybridisation, Polymerase Chain Reaction and p16 protein expression by immunohistochemistry. They concluded that a combination of ISH and PCR is superior to p16 IHC in detecting high grade cervical intraepithelial lesions(18). However, the cost effectiveness of each has to be borne in mind.

A study done by Deodhar et al in 2012 at Tata Medical Centre, Mumbai tried to identify the prevalence of subtypes of HPV in cervical lesions in rural Indian women. It was found that the overall prevalence of high risk Human Papilloma Virus in inflammatory / cervical intraepithelial neoplasia grades 1, 2 and 3 and invasive cancers was 37.6%, 63.5%, 97.2% and 92% respectively. 80.6% and 86.5% of CIN 3 and invasive cancers showed the presence of HPV 16 and 18. HPV was detected in 94.7% and 84.4% cases of invasive cancers and high grade intraepithelial lesions which showed diffuse strong positivity for p16 immunohistochemistry(19).

As the role of Human Papilloma Virus in cervical tumourigenesis was indisputably established, large number of studies were done to elucidate pathogenesis and to characterise the cell regulatory proteins which are disrupted due to the presence of virus. Moreover, direct viral detection, even with high sensitivity was expensive and hence cannot be routinely employed as a diagnostic tool in the general population. It will also require referral to tertiary care centres. Relying on histomorphology alone also

had its disadvantages in that it had substantial interobserver variability. It was logical to regard immunohistochemistry as an optimal confirmatory tool. Therefore, it became imperative to develop a surrogate marker for cervical dysplasia with sensitivity comparable to HPV DNA detection and specificity comparable to cytology.

In 2003, an elaborate study with respect to molecular markers in gynaecological oncology was done in Germany by Langosch et al. It was found that the main pathway of tumourigenesis was E6 and E7 viral protein expression leading to increased inactivation of tumour suppressor proteins p53 and Rb. This causes increase in the expression of cyclin dependent kinase inhibitor p16 which in turn causes inactivation of Rb due to negative feedback transcriptional control. Thus, cells proliferate with no CDK inhibitory effect in the presence of high levels of p16. High risk HPV infected cells also cause increased levels of cyclin E and cyclin dependent kinase 2, thus activating p21/p27-cyclin E/CDK2. It was found that there is down regulation of p21 and upregulation of p27 in most cervical premalignant lesions. It was also observed that there is over expression of CDC25A which activates CDK2. There is enhanced expression of proteins that control S phase, namely, cyclin A, cyclin B and CDC2. Out of all the proteins studied, p16 and cyclin E showed the most prominent upregulation(20).

The molecular basis for HPV induced carcinogenesis identifies various mechanisms that cause immortalisation of cervical epithelial cells (See Figure 3):

(a) Ability to produce genomic instability by causing monosomies and trisomies, chromatid breaks and gaps more commonly found in chromosomes 1, 3 and 5. The ability of the virus to integrate with the host genome is thought to be due to acquisition of chromosomal instability.

(b) Arrest of DNA damage response: this is thought to be due to interference with p53 mediated arrest of cell cycle and inactivation of p21. This causes accumulation of genetic changes which might increase the oncogenic potential of the virus.

(c) Ability of HPV to redirect suprabasal cells to continue DNA synthesis causing increased production of progeny viral genome through amplification.

(d) Continued expression of activated Ras which causes increased viral protein expression.

(e) Integration in HPV in proximity to MYC causing c-myc amplification which is observed more frequently in invasive cancers compared to benign and premalignant lesions.

(f) Increased expression of Her2 neu mRNA and protein, especially observed in case of adenocarcinoma

(g) Hypermethylation of gene promoters that cause down regulation of tumour suppressor genes

(h) the ability to interact with other infectious agents like Herpes Simplex Virus and Chlamydia trachomatis.(21)

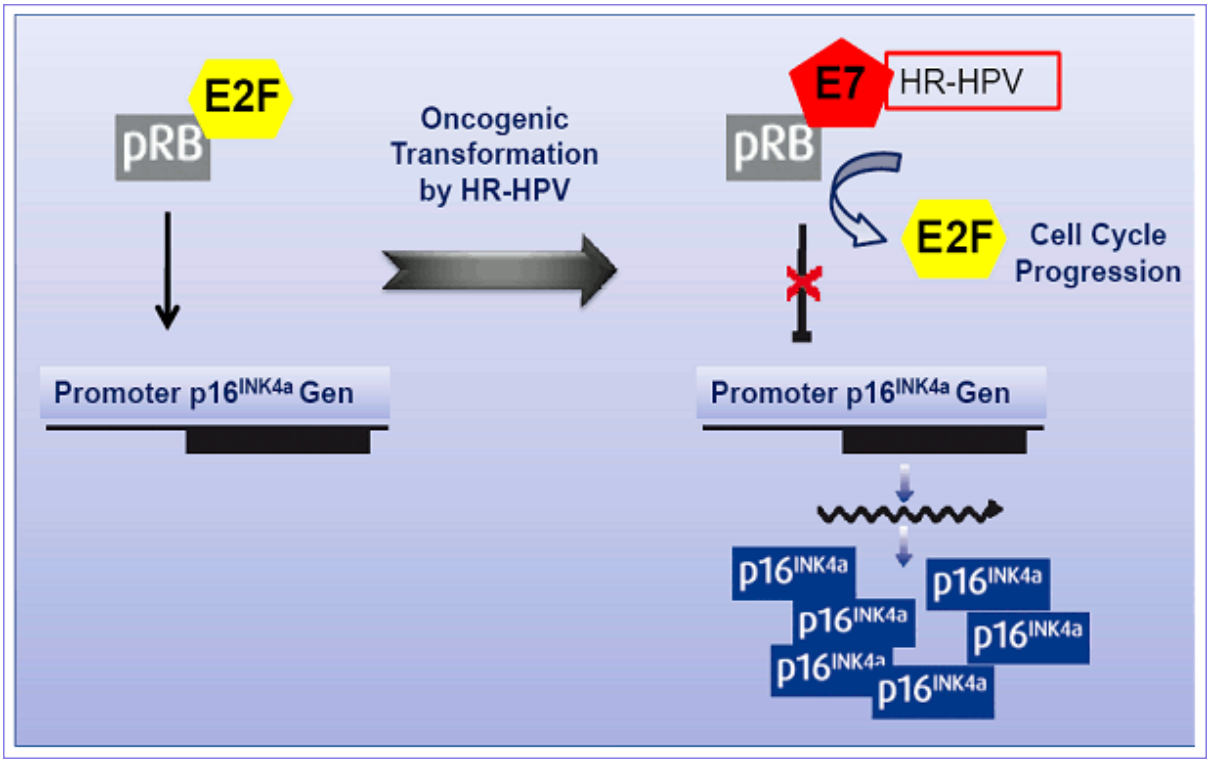

Figure 3: Mechanism of action of E7 protein and E7-Rb interaction

WHO states that loss of heterozygosity is observed in various chromosomes like 3p, 5p, 5q, 6p, 6q, 11q, 13q and 17q. The cases that had lymph node metastasis from cervical carcinomas had loss of heterozygosity at chromosomes 3p, 6p, 11q, 17p, 18q and X. Aberrant transcription, homozygous deletion and loss of heterozygosity of FHIT gene (fragile histidine triad) were also implicated in cervical tumours. Familial susceptibility for cervical cancers has also been observed, the largest family cluster obtained from Swedish family-Cancer database. The relative risk of developing cancer when daughter or mother was affected is 2. The familial predisposition is thought to occur due to involvement of genes that modulate immune response - HLA haplotypes(15).

Prognostic and predictive factors in cervical carcinomas:

Clinical parameters that are found to be significantly associated with worse prognosis are age, volume of tumour, vascular and lymphatic spread and stage of the disease. The

treatment is radiotherapy and surgery for tumours upto stage II A. External beam radiotherapy and intracavitary radiation therapy are given for stages II B to IV. A concurrent cisplatin based chemotherapy has shown to improve overall survival and disease free survival. The overall survival was increased by 16 % with combined chemoradiation therapy.

Histological parameters like grade and type of the tumour are also noted. The effect of grade on response to treatment is controversial. Adenocarcinomas, adenosquamous and neuroendocrine carcinomas are said to have a worse prognosis than squamous cell carcinoma as the progression time from premalignant lesions to invasive carcinomas was shorter .

Genetic factors: c erb 2 overexpression has a poor clinical outcome with increased chance of tumour recurrence. Amplification of c-myc protein is also associated with poor prognosis.

Cellular biomarkers of cervical carcinogenesis

In 2013, Tornesello et al aimed at identifying the cellular and viral biomarkers in premalignant cervical lesions that caused tumour progression. They analysed the candidate biomarkers, namely, HPV DNA, HPV E6 and E7 mRNA, HPV proteins, p16, MIB-1, MCM2 and TOP2A cellular factors and DNA methylation profile. The sensitivity of p16INK4A to identify CIN 2 or worse was 83.2% and 83.8% in ASCUS and LSIL cytology. The specificities were 71% and 65.7% for CIN2 and LSIL respectively. When dual staining strategy with p16/MIB-1 was employed, the sensitivity to detect ASCUS / LSIL increased to 92.2 / 94.2% respectively. The specificity rates were 80.6% and 68.0% for ASCUS and LSIL respectively. Thus p16 and MIB-1 when used in conjunction

showed a similar sensitivity and a higher specificity compared to any high risk HPV detection methods or p16 alone(22).

A detailed study regarding the structure of p16 and its regulatory mechanisms involved in carcinogenesis was done by Juan Li et al. p16 has a helix turn helix configuration and is composed of four ankyrin repeat motifs. It is situated in chromosome 9p21. It is a major negative controller of cell cycle. Apart from the most widely known pRB/E2F pathway, there are other alternate pathways by which p16 regulate cell cycle. p16 interacts with TFIIH, a general transcription factor required to form the pre initiation complex that inhibits phosphorylation of carboxy terminal domain (CTD) of RNA polymerase II thus causing cell cycle arrest. Additionally, p16 binds to glycine rich loop of c – jun – N - terminal kinases 1 and 3 and suppresses the kinase activity thereby interfering in cell transformation. p16 is also thought to be involved in cellular ageing as increased levels of p16 protein are present in aging human tissues in health as well as diseased states, although the exact molecular mechanisms have not yet been characterised. p16 deficient cells have shown to be sensitive to apoptosis induced by ultra violet radiation. Thus, p16 appears to be involved in the nuclear response of cells to genotoxic agents. Increased levels of p16 in tumour cells as a consequence of genotoxic DNA damage resulted in arrest of cell cycle, inhibition of apoptosis by preventing release of cytochrome c, depolarisation of mitochondrial membrane and activation of caspase cascade. p16 is the second most commonly mutated gene in human cancers, next to p53. The frequency of p16 inactivation in different types of human cancers are as follows: more than 85% in pancreatic carcinomas, followed by 50 – 70% of head and neck squamous cell carcinomas, approximately 70% of oesophageal cancers, 65% of colorectal malignancies, 65% of non small cell lung cancers, 60% of leukaemia, multiple myelomas and melanomas and 20% of breast carcinomas.

There are four types of genetic alterations that are thought to affect the function of p16: the most frequent changes are homozygous deletion and promoter hypermethylation. The other changes include loss of heterozygosity and point mutation. The type of genetic alteration varies in different types of cancers. Expression of p16 is regulated at various levels; post transcriptional modification occurs at sp – 1 binding site, HBP -1 binding site, ITSE, Ap – 1 binding site and PPRE sites. Post translational modification occurs by phosphorylation. The half life of p16 protein ranges from 30 minutes to three hours. It is degraded in a proteasome dependent manner. p16 is a member of INK4A family of proteins which also includes p15, p18 and p19. It is also called MTS -1 (Multiple tumour suppressor 1) (23).

Various studies from India have also tried to elucidate the genetic profile of cervical cancers. A study done by Rajkumar et al in 2011 observed up regulation of up to 20 genes and down regulation of 14 genes in cervical tumours. Real time PCR and immunohistochemistry were used and differential expression of each were analysed in normal, CIN 1 / 2, CIN 3 and invasive squamous cell carcinomas. Following genes were upregulated in CIN 3 relative to normal and CIN 1 suggesting a role in early tumour transformation: UBE2C, NUP210, MELK, CCNB 1, CCNB 2, PLOD 2 and CDC 20. Other genes that were over expressed were: IL8, INDO, AGRN, DTXL, ISG 15, ISG 20, MMP 1, MMP 3, CCL 18, STAT 1 and TOP2A (24).

Another study conducted in Tata Memorial hospital, Mumbai by Asha Thomas et al studied differential expression of various genes through progressive FIGO stages in Indian women. Stages I to IIA were considered early and IIB onwards as late stages. They observed that SPP-1 (Secreted Phosphoprotein 1) more commonly known as osteopontin, had shown 34.6 times overexpression in cervical carcinoma. In terms of its

use as a diagnostic marker, 50.6% sensitivity and 95.0% specificity were obtained. It also correlated significantly with overall survival and disease free survival (p values 0.002 and 0.033 respectively). It was also noted that there was increasing expression of PCNA (Proliferating cell nuclear antigen) through progressive grades of dysplasia. There was down regulation of apoptosis related genes like *IP3*, *BCL2L11*, *TNFSF13*, *PERP*, *ATF6*, *BPT* and phosphatases *DUSP1*, *PPP2R5E*, *PTPN4* were underexpressed. The genes involved in transcriptional activation, ATF2, *GTF3C1*, *TFE3*, *MCRS1* and signalling and migration of cells (*MAPRE3*, *PAK and PIK3R1*) were overexpressed in advanced stage as compared to early-stage cervical cancer (25).

A review was done by Gupta et al on biomarkers in cervical tumours. The sensitivity and specificity of p16 immunohistochemistry to detect high risk HPV were found to be 84 % and 98 % respectively. The positive predictive value was 97 % and negative predictive value was 86 %. High risk HPV positivity was detected in 93 % of p16 positive CIN 1. Widespread expression of p53 and p21 were seen in low risk HPV subtypes. p53 is thought to be a predictor for rate of regression in CIN 3 cases. However, Retinoblastoma protein expression is considered a stronger predictor for progression of cervical intraepithelial lesions into a higher grade. It was also demonstrated that addition of CK 13 and CK 14 to the panel of markers helped to identify the subgroups that had a higher risk of progression. Telomerase activity did not reflect rate of progression. Loss of FHIT expression was associated with increased rate of progression. ProExC which is a combination of minichromosome maintenance 2 and topoisomerase IIA were found to be a better positive predictor for rate of progression than p16. MCM5 intensity did not correlate with the presence of high risk HPV infection, thus making it a valuable biomarker for HPV independent cervical dysplasia. CDC6 was not detected in low grade

cervical lesions, thus restricting its use only for the detection of high grade lesions and invasive carcinomas. Thus, it was concluded that a combining p16, ProExc and MIB-1 proliferation index along with high risk HPV DNA detection formed the best panel to detect lesions that would progress (26).

One of the major challenges in the interpretation of cervical biopsies is to distinguish benign and reactive conditions from dysplastic lesions. There are various benign mimics of neoplasia. The most notable amongst them is radiation induced changes which can be easily mistaken for neoplasia. Complex architecture and cribriform patterns with nuclear hyperchromasia and enlargement may be present even after 18 years of completion of radiation therapy. However, the molecular and immunophenotypic features described above are usually absent.

Acute and chronic cervicitis may mimic carcinoma on colposcopic examination due to their congested and inflamed appearance. Various microorganisms, most commonly Trichomonas vaginalis, Chlamydia trachomatis, Herpes simplex, bacterial vaginosis can cause marked inflammation and reparative changes which can resemble carcinoma.

Cervix is also a site for the development of various metaplastic lesions. Squamous metaplasia is so consistent a finding that it is considered a normal physiological response to ectropion. Tubal, endometrioid, intestinal and transitional cell metaplastic changes are the other conditions that can occur in cervix.

The glandular compartment can also undergo various reactive changes which can confuse inexperienced pathologist. Notable amongst them is microglandular hyperplasia and endocervical tunnel clusters. Microglandular hyperplasia consists of small closely packed glandular structures lined by columnar cells with bland nuclei.

Endocervical tunnel clusters are small endocervical glands arranged in a lobular configuration (14).

Thus cervical lesions range along a spectrum of benign and reactive conditions through increasing grades of CIN 1, 2 and 3 and invasive squamous cell carcinomas. The endocervical lesions encompass reactive conditions like microglandular hyperplasia, cervical glandular intraepithelial neoplasias, low grade and high grade and invasive adenocarcinoma. The fact that these lesions progress over a fairly well defined time period with most of the lesions demonstrating a definite viral etiology make them potentially preventable. Various methods were tried and tested for early detection of premalignant lesions. The evolution of Pap test was a historical landmark which proved to be a highly effective screening tool which drastically reduced mortality due to cervical cancer in developed countries.

Pap test was discovered and named after Georgios Papanikolaou in 1928. The development of exfoliative cytology and routine use of Pap stain began in 1941. Conventional smears and liquid based cytological smears are commonly used. The Bethesda system was introduced in 1988 and is the most widely accepted method for reporting cervical cytology specimens to ensure uniform terminology. It has been revised in 1991 and 2001 incorporating various modifications.

The current recommendations for cytological screening vary in different countries depending upon the prevalence and incidence of the disease. In general, the first cervical cytology should be performed at 21 years of age or within 3 years of first sexual intercourse and repeated every 3 years. After 30 years of age, women with negative cytology and absent HPV infection should be screened every 5 years. Pap test should be repeated every 6 to 12 months in women who harbour high risk HPV even in the

presence of normal cytology. If cytological abnormality is detected on Pap test, the patient is advised to undergo colposcopic biopsy. Low grade lesions may be followed up; however, ablative cryotherapy is advised, especially in cases where the patients are unlikely to come for follow up. High grade lesions should be treated with conisation. Pelvic and abdominal imaging should be done for invasive carcinomas to ascertain the stage of disease (27).

Briefly, the Bethesda system of classification 2001 is based on the severity of dysplasia of intraepithelial and glandular lesions. The adequacy of the specimen needs to be mentioned. It is classified into broad categories: negative for intraepithelial lesion / malignancy, epithelial cell abnormality - squamous and glandular.

Epithelial cell abnormality, squamous is further classified into low grade and high grade squamous intraepithelial lesion and squamous cell carcinoma. The equivocal category, Atypical Squamous Cells is further divided into “Undetermined significance” and “cannot exclude HSIL”. Epithelial cell abnormality, glandular is divided in to Atypical glandular cells – endometrial, endocervical, not otherwise specified or favour neoplastic, Adenocarcinoma in situ and Adenocarcinoma (28).

The Bethesda system estimates the accuracy rate of LSIL to be 80 %. Of the remaining 20 % which are misinterpreted, 33 % belong to a high grade category.

According to a study done by Nguyen et al, the Bethesda system of reporting has a specificity of 90-99% and around 85% sensitivity to detect cervical dysplastic lesions. The false negative rate was found to be approximately 20% and was attributed to screening error and sampling error(29).

Denny et al compared various screening techniques for cervical neoplasia in developing countries including India in terms of sensitivity, specificity, availability and cost effectiveness. It was observed that at a given point of time, 75 % of women had undergone cervical screening at least once in the last 5 years in developed countries compared to less than 5 % in developing countries. It was found that cytology had a high specificity (91 - 96 %) more than that of visual inspection methods with acetic acid and Lugol's Iodine (45 – 86 %) and moderate sensitivity (44 – 78 %) compared to HPV DNA testing (66 - 100 %). The study concluded that incorporation of visual inspection methods along with cytological screening in India would improve the detection rate of cervical abnormalities. The Government of India has formed a task force to ensure the introduction of visual inspection method by acetic acid in 50 districts in the next 5 years (30).

Another study done by Ansari et al in 2011 focussed on the utility of Pap smear in detecting the distribution of premalignant and malignant lesions of cervix in premenopausal women. The overall sensitivity of Pap test in detecting high grade dysplasia was 70-80%. It was also observed that there was increasing incidence of high grade dysplasia with increasing age. The established risk factors like multiple sexual partners, early age of sexual intercourse and concurrent sexually transmitted infections were found to be significantly associated with cervical dysplasia. HPV infection screening showed a higher sensitivity compared to cytology. However, it was concluded that in Indian setting, cervical cytology was a more cost effective and efficient tool to detect cervical dysplasia (31).

When cytology and HPV testing were compared in American population, Pap smear and HPV test had a sensitivity of 55.4 % and 94.6 % respectively and specificity was 96.8 %

and 94.1 % respectively. Both the tests had a negative predictive value of more than 99 % (32).

An epidemiological study done in Patiala evaluated 300 routine Pap smears. 45.3 % of the patients were between 31 to 40 years of age. 59 % of the patients presented with vaginal discharge. The mean age of women diagnosed to have LSIL was 32.3 years, HSIL, 40.5 years and invasive carcinoma 57 years. The most common clinically visible lesion in patients with squamous intraepithelial lesion and invasive carcinomas was erosion (35.7 %) followed by cervix that bleeds on touch (28.6 %). 5 % of the smears were found to have epithelial abnormalities. The most common reactive condition was non specific inflammation (71.3 %) followed by Gardenerella (2.7 %). LSIL was detected in 3.4 %, ASCUS in 0.3 %, HSIL in 0.7 % and squamous cell carcinoma in 1.3 % (33).

Women diagnosed with low grade lesion may present later with an invasive carcinoma. Therefore a study was done by Bofin et al which re-examined all the previous Pap stained slides of patients diagnosed with invasive squamous cell carcinoma. It was observed that first High Grade squamous lesion could have been diagnosed 4.2 years earlier than originally thus emphasising the need for meticulous examination and frequent screening compared to current guidelines (34).

A study done by Moy et al tried to establish the efficacy of cervical cytology as a primary screening modality in Chinese population. The sensitivity and specificity of three modalities were compared, namely, (1) HPV DNA testing, (2) Pap test and (3) cotesting, i.e. both (1) and (2). The highest sensitivity was observed for cotesting strategy (99.4%). HPV DNA test alone had a sensitivity of 96.3% followed by Pap alone (80.2%). The highest specificity was observed for Pap test alone (93.3%) followed by HPV DNA (85.5%) and cotesting strategy had a specificity of 84.8 %. Among the single test

strategies, the sensitivity of HPV DNA was higher compared to Pap test alone. However, the specificity was higher for Pap test alone with a lower number of cases referred for colposcopy (35).

One of the major drawbacks of Pap smear reporting was variable specificity and a relatively low sensitivity reported in different laboratories. The reasons for low sensitivity for cervical smear were studied by Farimani et al by evaluating 1224 smears. The reasons for low detection rates were classified into three categories: Errors while sampling (11.7 %) which included improper endocervical cell sampling, low density of cells, improper fixation and obscuring inflammation, errors in preparation of cytological smears (74.5 %) such as poor staining and various artefacts and errors in interpretation (13.8 %) when there was discordance in interpretation among the cytologists (36).

Therefore, a newer modality was tried by the Zetiq cell detect staining kit which consists of a basophilic green stain, acidophilic red stain and a proprietary plant extract. The sensitivity of Thin Prep was 84.4% and that of the cell detect system was 90.6%. The specificity of Thin Prep was 80.0% and that of the cell detection system was 76.3%. Thus, Thin Prep was superior to Zetiq in terms of specificity (37).

A quantitative survey was done to compare the performance of Thin Prep liquid based cytology and conventional Pap smears. The two methods had a concordance of 89 % and 92 % on five level and dichotomous classifications. When compared with histology, the sensitivity rates of Thin Prep and conventional smears were 76 % and 68 % respectively. The specificity rates were 86 % (Thin Prep) and 79 % (conventional smears) (38).

Another elaborate study was conducted by Renshaw et al to detect sensitivity in gynaecological cytology. The study also aimed at analysing various ways to increase the

sensitivity, namely, rescreening of conventional smears, second thin layer preparation, repeat Pap smear at a subsequent time and biopsy. The overall sensitivity of Thin Prep was 50-75%. On addition of rescreening strategy, the sensitivity increased to 84%. The sensitivity increased to 90% with second thin layer preparation and repeat Pap smear. This was thought to be due to interobserver variability (28-72%) and technical reasons such as difference in quality of staining. Therefore, the study confirmed the use of cervical cytology as a primary screening tool and proposed the development of a surrogate marker for a more objective evaluation (39).

The test limitations and errors of interpretation of cervical cytology were analysed in a study done by Ronco et al. The sensitivity of cytologists in detecting HSIL was 100% and LSIL was 67% when the slides were reviewed by expert supervisors. Biopsy cytology correlation was also assessed and it was found that 88% of CIN 2/3 cases and 74% of CIN 1 cases were detected by cytologists in Pap smears (40).

Another study tried to demonstrate if there was an additional benefit if a repeat Pap smear was performed at the time of colposcopy in detecting high grade lesions. The Pap smear and biopsy results were independently reviewed and a repeat Pap smear was performed at the time of colposcopy. It was found that only 2% of the patients had significant change in management as a result of repeat smear. It was concluded that Pap smear done at the time of colposcopic biopsy did not yield a better result compared to the diagnostic smears that were taken earlier (41).

Follow up with biopsy and high risk HPV testing was done by Faye et al in all cases diagnosed as HSIL on cytology. 96.0% cases of HSIL were positive for high risk HPV. 72.3% of high risk HPV positive HSIL cases, on biopsy showed CIN 2/3, whereas only 33.3% of high risk HPV negative HSIL cases showed CIN 2/3, raising the possibility of

benign mimics of HSIL. It was also observed in this study that incidence of high risk HPV reduced slightly with increasing age (42).

In a cytohistological study done in St Joseph's health centre, Canada, the sensitivity of cytology in detecting cervical dysplasia was 88.5%. 100 ASCUS cases were studied. In case of ASCUS, favour dysplasia, the sensitivity was 73% and ASCUS favour reactive it was 27%. Among the cytological features assessed, nuclear membrane irregularity was the only feature predictive of dysplasia. The interobserver agreement was 0.41 (43).

In a study done by Aoyoma et al, 37 cases of ASCUS were studied with histopathological and immunohistochemical follow up. The features taken into account were loss of polarity, absence of perinuclear halo, mitoses, presence of primitive cells in the upper two thirds and positivity for p16 and MIB-1 in the upper two thirds. 84.0% of neoplastic cases had 5 or more of these features. 89.0% of the non neoplastic cases had less than 2 of these indicators (44).

Once the use of Pap smear was established in detecting squamous lesions, attention was shifted towards detecting efficacy of pap testing for endocervical lesions. Another potentially troublesome and diagnostically challenging area for cytologists was "atypical squamous cells of undetermined significance". This category caused a management dilemma for the clinicians as well. Therefore, a number of studies were done to improve the sensitivity of Pap tests. The next step was the advent of colposcopy. Colposcope is a binocular instrument that gives a 6 to 40 fold magnification. 4-5 % acetic acid is applied onto the cervix and acetowhite epithelium is assessed. The colour, contour, margins, iodine staining and arrangement of blood vessels are noted. Sharply delineated and opaque areas which persist for several minutes are characteristic of cervical

intraepithelial lesions and cervical carcinoma. It has been observed that glandular lesions cause more subtle changes.

A study done in India observed that amongst cytology and visual inspection of cervix with acetic acid and Lugol's iodine, cytology alone had a higher specificity and sensitivity. The sensitivity of visual inspection with acetic acetic acid, Lugols iodine and cytology were 64.5%, 64.5& and 67.7% respectively and specificity were 84.2%, 85.5% and 95.4% respectively. In addition, p16 positive CIN 3 (93.8%) and CIN 2 (76.9%) were positive on cytology as opposed to visual tests (68.8% and 53.8%) proving that cytology had a higher sensitivity to detect dysplasia of cervix (45).

In a study done by Phongnarison et al it was found that 36.4 % of women were diagnosed to have HSIL and 5 % had frank carcinoma when colposcopic biopsy was done following a diagnosis of LSIL. Hence the authors advise that in areas with high incidence of high grade squamous lesions, women with low grade lesions require an immediate colposcopic examination to rule out a higher grade lesion (46). A study done by Dolman et al also suggested that a repeat Pap smear done at the time of colposcopic examination is beneficial in women who have been diagnosed with high grade squamous lesions (47).

A study was done in New York School of medicine to evaluate the significance of "endocervical crypt involvement" in HSIL. It was concluded that the diagnosis of endocervical gland involvement on Pap smears did not show increased frequency of glandular involvement on follow up biopsies (48).

It is thought that the presence of endometrial cells in Pap smears of women above 40 years of age is abnormal. However, a study done by Thrall et al showed that women

with endometrial cells in Pap smears did not have an increased incidence of endometrial hyperplasia or malignancies (49).

Pap smear findings in cervical glandular lesions was extensively studied by Chengquan Zhao et al, 61.6 % had glandular cell lesions as the most common recent abnormal Pap smear finding. 38.4% had squamous cell abnormalities alone and 60% had coexisting cervical intraepithelial squamous neoplasia. 61.4% had a negative earlier Pap test taken within 4 months to 3 years. 97.2 % of the cases tested positive for Human Papilloma Virus (50).

The rate of false positive histology according to colposcopic referral was studied by Palma et al. All CIN 1 and CIN 2 cases were blindly reviewed by two pathologists. The specificity and sensitivity of colposcopy guided biopsy were 98 % and 84 % respectively. Unnecessary treatment was given for 27% of women who had ASCUS, 8 % of women with LSIL and 10% of women who were HPV positive with cytological lesions in Pap test. (Any patient whose diagnosis was downgraded to a low grade lesion on biopsy was considered to have received unnecessary treatment) (51).

The variable sensitivity and specificity for Pap smear as well as subjectivity of evaluation thus leading to a high rate of unnecessary treatment lead to the quest for a surrogate marker for a more objective evaluation. Duncan et al studied the use of p16 as a diagnostic aid in cases of ASCUS on Pap smear.p16 immunohistochemical stain was performed on Thin Prep smears diagnosed as ASCUS by 5 cytopathologists. It was followed up with tissue diagnosis, repeat smears and hybrid capture 2. It was demonstrated that p16 scores had a better correlation with tissue follow up than hybrid capture 2. The positive predictive value and sensitivity of p16, compared to tissue follow up were statistically more favourable. However, p16 expression was consistently

present in atrophic Pap smears, thus limiting its use alone in triage of atypical atrophic smears (52).

It has been commonly observed that discordance exists between cervical punch biopsies, Pap smears and conisation specimens. The most common diagnostic error that has been noticed is the absence of dysplasia in conisation specimens when dysplasia has been reported in biopsies. Carrigg et al studied 53 negative conisation specimens with review of their prior biopsy and cytology.p16 immunohistochemical stain was performed on all the specimens. 26 % of conisation specimens were detected to have dysplastic lesions. 28 % of them were true negatives and the previous biopsies had overdiagnosed HSIL. However, 45 % of cones were actually negative but had HSIL in the pre surgical material. Amongst these 11 % showed evidence of dysplasia in subsequent biopsies and 26 % were negative on follow up. It was concluded that to prevent over diagnosis of HSIL on Pap smears and cervical biopsies, deeper level examination and use of p16 immunostain may be helpful. The causes for this discrepancy were thought to be sampling error, interpretation error, incomplete tissue examination, spontaneous regression of lesion and probably complete excision of a small focus of HSIL (53).

Various studies have been done in different parts of the world to investigate the role of p16 as a surrogate marker in detecting cervical dyskaryosis. The diagnostic accuracy of p16 alone and in conjunction with other markers in cervical lesions has been extensively studied on Pap smears and cervical biopsy specimens for intraepithelial and invasive squamous lesions and glandular lesions.

The ability of p16 to discriminate between CIN 1 lesions and non neoplastic equivocal epithelial lesions of cervix was studied by comparing 81 cases of CIN 1 with 52 cases of squamous metaplasia, 33 cases with HPV related cellular changes, 4 cases of

microglandular hyperplasia and 12 cases of chronic cervicitis. It was observed that none of the reactive conditions demonstrated positivity for p16. 19 cases of CIN 1 showed strong positivity and 4 cases showed focal positivity for p16. Of the remaining 58 CIN 1 cases, 50 cases did not stain with p16 and were negative for HPV DNA on PCR (54).

Yoshida et al in 2004 studied the usefulness of p16 expression in liquid based cervical smears and tissue sections and correlation with HPV DNA status. A total of 98 cases were studied, of which 38 cases were ASCUS, 12 cases LSIL, 33 cases HSIL and 15 cases frank carcinoma. It was found that the rate of concordance between cytological and histological diagnosis was higher in high grade dysplastic lesions than low grade lesions. 16 cases showed marked discordance between cytological and histological diagnosis. Diffuse and strong staining was observed in all HSIL and invasive carcinoma cases. Only two adenocarcinoma cases were negative for p16. 60 cases demonstrated positivity for high risk HPV and 55 cases out of 60 showed strong p16 positivity (55).

Sano et al studied the expression pattern of p16 and retinoblastoma protein in cervical intraepithelial lesions as both the proteins are important components of cell cycle regulation. Out of 98 cases of cervical dysplastic lesions studied, all the cases showed strong nuclear and cytoplasmic positivity for p16. Scattered nuclei of normal as well as neoplastic cells showed Rb protein expression. Therefore, the study suggests that combined mutational inactivity or deletion of Rb and p16 proteins is unusual (56).

A recent study published in May 2014 by Burgeron et al reinforces the advantage of p16 immunohistochemistry over Pap smear testing which has low sensitivity and HPV testing which has a low specificity. The sensitivity and specificity of p16 cytology in ASCUS were found to be 92.6 % and 63.2 % respectively. When used in combination with MIB-1 proliferation index, the specificity increased to 80.6 %. However, the

sensitivity of HPV DNA was found to be 90.1 % and the specificity was 37.8 %. This proves the fact that all HPV colonised epithelial cells do not necessarily undergo neoplastic transformation. Therefore the study suggests that use of p16 alone has reasonably high sensitivity and specificity and can be used as a screening tool (57).

p16 expression was evaluated in Thin Prep smears and cervical biopsy samples along with HPV detection and typing by Murphy et al. p16 expression was found to be negative in all normal cases. It was positive in all dysplastic glandular and squamous lesions, except one CIN 3 case. The staining pattern was predominantly nuclear in CIN 1 cases, whereas both nuclear and cytoplasmic in CIN 2, CIN 3, cGIN and invasive carcinomas. All cases that were positive for HPV DNA showed a diffuse and strong staining for p16, however, all cases which showed p16 positivity were not positive for HPV DNA, thereby suggesting that other oncogenic pathways play a role in cervical neoplasia. p16 intensity was lower in cervical tissue harbouring low risk HPV. 10 % of normal cervical epithelium showed positivity for low risk HPV (HPV 6/11). HPV 16 was positive in all cases of cGIN. 72% if CIN 1 and 78 % each of CIN 2 and CIN 3 were positive for HPV 16. HPV 18 was positive in 4% of CIN 2 and 10 % of CIN 3. All the invasive carcinomas were positive for HPV 16. Thus HPV 16 was found to be the most prevalent oncogenic strain (58).

The major drawback of interpretation of cervical dysplasia is the lack of interobserver agreement. It is also possible that a small focus of dysplasia in otherwise reactive epithelium maybe missed even by an experienced pathologist. A study done by Klaes et al assessed the difference in interobserver agreement in Haematoxylin and eosin stained sections and immunohistochemical stain for p16 using kappa statistics. The opinions of five experienced pathologists were sought. It was found that significant

discordance exists between interpretations of tissue sections which were particularly high in case of low grade lesions (kappa value 0. 60). However, the agreement was better in interpreting p16 expression (kappa value 0. 91). It was also observed that p16 expression was limited to dysplastic epithelium in CIN 1, 2 and 3 lesions clearly delineating adjacent reactive epithelium and thus enabling the pathologist to detect even a small focus (4).

A study done by Karcheva et al divided the cervical lesions into 4 categories: non dysplastic lesions which include immature squamous metaplasia, atypical squamous metaplasia, inflammation and inflammatory atypia., increasing grades of cervical intraepithelial neoplasia, invasive squamous cell carcinoma and glandular lesions including microglandular hyperplasia, endocervical dysplasia and adenocarcinoma. There was statistically significant association between p16 expression and dysplastic lesions. None of the reactive lesions stained positive for p16, thus making it an independent and reliable marker for cervical dyskaryosis (59).

A systematic review done by Tsoumpu et al established the use of p16 as an adjunct marker for cervical dysplasia in cytological and tissue specimens. It was observed that the proportion of cervical Pap smears expressing p16 increased with increasing severity of dysplasia. 12 % of the normal smears, 45 % of ASCUS and LSIL and 89 % of HSIL cases showed diffuse and strong positivity for p16. In tissue sections, 2% of normal biopsies, 38 % of CIN 1, 38 % of CIN 2 and 82 % of CIN 3 showed strong expression(60).

Another study done in Germany investigated the accuracy of p16 in detecting high grade lesions on 500 tissue sections of cervical punch biopsies and conisation specimens. Dichotomised gold standard was established by consensus of 3 expert cytopathologists. There was a significant improvement in diagnostic accuracy when p16

immunostain was added (p value = 0.0004). The sensitivity to detect high grade lesions increased by approximately 13 %. False negative results were reduced by half. There was significant increase in the interobserver agreement which increased from 0.566 to 0.749 (p value = 0.001). There was excellent reproducibility as well (kappa value = 0.899). Thus the study concluded that the use of p16 would aid as a diagnostic tool in detecting cervical dysplasia due to improved interobserver agreement(61).

A tissue microarray study on 796 specimens was done by Lesnikova et al. Tissue microarray is a technology used to study gene expression in tissue sections. Small cores of tissue are removed from the paraffin blocks and arrayed. Multiple samples can be stained simultaneously for a single biomarker or immunohistochemical stain. One advantage of this study was that all samples undergo staining with same protocol thus ensuring uniformity. Two methods of analysis were used: (1) Simple method – evaluated as positive or negative. (2) Semi quantitative method- Scoring system based on intensity and proportion of staining. Normal cervical tissue did not stain for p16. However, 72.3 % of CIN 1, 91.0 % of CIN 2, 98.3 % of CIN 3 and 98.5 % of invasive carcinomas showed positivity for p16. Both the scoring systems were compared. The semi quantitative system gave a detailed account of the spread of dysplasia, however overall performance of both the systems was comparable. It is known that many of the low grade lesions undergo spontaneous regression. Since transcription of E7 protein of HPV is required for upregulation of p16, it was suggested that low grade lesions expressing p16 are more likely to undergo transformation into high grade lesions (3).

Diagnostic value of p16 in preneoplastic and neoplastic lesions of squamous and glandular epithelium was studied by Izadi Mood et al. 81.8 % of low grade CINs, 91 % of high grade CINs, 90 % of squamous cell carcinomas and 75 % of adenocarcinoma

strongly expressed p16 protein. 10 % of normal cervical tissue showed p16 positivity. When the relationship between reaction intensity and lesion severity was compared, both were found to be significantly different in different histological subtypes. However, reaction intensity was proved to be superior in logistic regression model, thus making it the best parameter to assess p16 expression (p value < 0.005) (62).

The expression pattern of p16, MIB-1 and HPV viral load were used to predict residual disease in conisation specimens with positive margins. The expression of p16 and MIB-1 had significant association with grade of CIN (p values = 0.0012 and 0.0006 respectively). In both univariate and logistic regression analysis, the following parameters did not have significant association with residual disease after conisation with positive margins: Age, parity, cytology, grade of the lesion, load of HPV and expression of p16 and MIB-1 (p value > 0.05). However, HPV viral load showed significant differences with expression of p16 and MIB-1 (63).

A study done in Gunma University, Japan, tried to use p16 expression pattern to differentiate between premalignant and malignant lesions of cervix and condyloma acuminatum, which is a benign HPV induced change in cervical epithelium. Correlation with HPV subtyping was also done. It was found that there was diffuse and strong positivity for p16 in high grade premalignant and malignant lesions caused due to high and intermediated grade HPV, namely HPV 16, 18, 31, 33, 52 and 58. Low grade lesions and Condyloma acuminata showed absent or weak staining. This is thought to be due to difference in functional inactivation of retinoblastoma protein by different strains of viruses. Thus, it enables the distinction between low grade and high grade HPV infections (64).

Once the role of p16 in squamous lesions was established, focus was shifted to glandular lesions. The emphasis on glandular lesions also increased as a wider spectrum of endocervical lesions were described by McCluggage. He put forward that a spectrum of benign, premalignant and malignant lesions exist in the glandular compartment. The role of HPV in neoplastic progression was also increasingly becoming clearer. Relatively newer entities were also described which pose a diagnostic challenge to the pathologist such as typical and atypical lobular endocervical glandular hyperplasia, tunnel clusters, adenoma malignum and adenocarcinoma, gastric type. It was also pointed out that tubulosquamous polyp, derived from paraurethral Skene's glands can also mimic a low grade adenocarcinoma. Even though adenocarcinoma in general has a poorer prognosis compared to squamous cell carcinoma, evidence suggests that early adenocarcinoma has a comparatively better prognosis and is amenable to conservative management. Since it is essential to establish the site of primary tumour, it was imperative to have a marker which gives reasonably high sensitivity and specificity to establish cervical origin. The conventional panel of markers, namely, estrogen and progesterone receptors, CEA and Vimentin had their pitfalls especially in case of low grade adenocarcinoma. It was also noted that there is increasing frequency of association between cervical neuroendocrine neoplasias and glandular neoplasias(65). Colposcopy was also not as effective in detecting glandular lesions compared to squamous lesions.

The expression profile of p16 was studied in adenocarcinoma and its precursors and benign lesions of endocervical glands by Negri et al in 2003 in tissue sections and Thin Prep smears. HPV DNA detection was done using Hybrid capture 2. A total of 45 cases were studied: 18 invasive, 8 adenocarcinoma in situ, 4 atypical glandular lesions and 15 reactive conditions of endocervix. All cases of invasive as well as in situ adenocarcinoma demonstrated strong and diffuse positivity for p16. Four cases of atypical glandular

lesions showed only focal expression. All reactive conditions showed negative staining. In Thin Prep smears, all neoplastic endocervical cells showed strong positivity. All the high grade lesions and carcinoma were associated with high grade HPV (66).

Concomitant p16 expression along with MIB-1 proliferation index and HPV detection was used to distinguish between reactive glandular lesions and early endocervical glandular neoplasia. It was found that strong and diffuse expression of p16 along with moderate to high MIB-1 proliferation index was observed in cases of adenocarcinoma in situ. All the cases were positive for high risk HPV as well. However, a weak staining for p16 was observed in endometrial epithelium and tubo-endometrial metaplasia. They were negative for high risk HPV and had a low MIB-1 index. Thus, the authors suggest the use of MIB-1 and high risk HPV detection in ambiguous cases (67).

A study from Netherlands done by Zeilinski et al used a combination p53 and p16 with high risk HPV detection. Atypical glandular lesions are not as frequently diagnosed as squamous lesions in Pap smears. Moreover, it is diagnostically challenging to distinguish endocervical adenocarcinoma from endometrial carcinomas. It was proposed that incorporating immunohistochemical markers and high risk HPV testing may improve the detection rate of atypical glandular lesions in screening programmes. 65 cases of adenocarcinoma in situ and 77 cases of adenocarcinoma were studied. High risk HPV was detected in all cases of in situ carcinomas and 94 % cases of cervical adenocarcinoma. None of the 20 endometrial adenocarcinoma demonstrated HPV positivity. HPV 18 was the most prevalent subtype and was present in 55 % of cervical adenocarcinoma and 68 % of in situ carcinomas. Strong and diffuse staining for p16 was noted in 95 % of high risk HPV positive adenocarcinoma (p value <0.001). Significant association was present between p53 immunostaining which marks stabilised wild type

or mutant p53 protein and high risk HPV. However, there was no difference in staining pattern of p53 and p16 in HPV negative adenocarcinoma and endometrial adenocarcinoma. As only a very few cases of cervical adenocarcinoma were negative for HPV, it was suggested that p16 immunostaining would be a useful marker to establish a primary endocervical adenocarcinoma (68).

HPV detection pattern and p16 expression were studied in unusual variants of adenocarcinoma and compared with usual type by Houghton et al. A total of 63 cases were studied which included 43 usual type, 3 gastric type, 3 intestinal type, 4 clear cell type, 3 mesonephric type, 2 serous type, 1 hepatoid type and 4 minimal deviation type. All cases underwent immunohistochemical staining with p16 and HPV genotyping. 57 % of all cases showed HPV positivity. The commonest subtypes were HPV 16 and 18. All cases of usual type and single serous carcinoma were positive for HPV. Rest of the unusual type cases were negative for HPV. 42 out of 43 usual type cases showed strong and diffuse positivity for p16. 2 out of 4 minimal deviation type, 2 out of 3 gastric type and 1 out of 2 serous carcinoma showed strong p16 staining. 1 out of 3 intestinal type, 2 out of 3 mesonephric type and 1 out of 4 clear cell type showed focal p16 staining. Thus it was concluded that p16 positivity in unusual subtypes in the absence of high risk HPV is attributed to retinoblastoma inactivation by some mechanism other than HPV (69).

p16 expression was used to discriminate between adenocarcinoma of endocervical and endometrial origin. p16 immunohistochemical stain along with HPV DNA detection was done in 24 cases of endometrial and 19 cases of endocervical adenocarcinoma. p16 was strongly expressed in 18 endocervical adenocarcinoma cases, out of which 14 were positive for HPV DNA. In contrast, endometrial carcinomas showed a patchy and weaker staining for p16 and all the cases were negative for HPV DNA (5).

A number of studies were done in various centres in India to assess demographic difference in HPV prevalence and p16 expression in cervical dysplastic lesions. A study done by Tripathi et al in 2003 documented various genetic alterations in p16 locus in women with cervical dysplasia. It was found that 15 out of 46 cases (33 %) showed alterations in p16 gene. Mutations were present in 7 out of 46 cases (15 %). Most of the mutations occurred in exon 2, one mutation at intron 1 / exon 2 splice junction and three were silent mutations. Homozygous deletion and promoter hypermethylation were detected in 4 out of 46 cases (8.7 %) and 3 out of 46 cases (6.5 %) cases respectively. Microsatellite size changes and loss of heterozygozity were detected in 8 out of 46 (17 %) of samples. It was also found that high risk HPV (HPV 16/18) was present in 35 out of 46 (76 %) samples. There was no significant association between HPV infection and p16 alterations (70).

Another study done in PGIMER, Chandigarh assessed p16 expression in 20 cases each of normal cervical epithelium, CIN 1, CIN 2, CIN 3 and invasive carcinomas. It was found that 18 out of 20 normal cases were negative for p16. Only 20 % of CIN 1 cases showed p16 positivity. 45 %, 55% and 95 % of CIN 2, CIN 3 and invasive carcinomas showed p16 positivity. The statistical difference between p16 expression patterns was found to be significant between CIN 2, CIN 3 and invasive carcinomas. (p values = 0.021, 0.008 and 0.000 respectively) (71).

A study done in Regional Cancer Centre, Thiruvananthapuram, assessed the association between p16 expression in cervical neoplasia and presence of HPV DNA in southern India. A total of 177 cases were studied out of which 42 were classified as benign, 34 as LSIL, 48 as HSIL and 53 as invasive carcinoma. 32 out of 42 benign cases were negative for p16. In LSIL category, 28 out of 34 cases were positive for p16 protein. 45 out of 48

cases of HSIL and 50 out of 53 invasive carcinomas showed strong diffuse staining pattern. HPV DNA was absent in all the benign lesions. HPV DNA was detected in 47 cases, of invasive carcinoma, 37 HSIL and 3 LSIL samples. Of these, high risk subtypes, HPV 16/18 were found in 37 HSIL and 42 carcinoma cases. Low risk subtypes, HPV 6/11 were found in 3 LSIL and 1 carcinoma samples. 1 carcinoma case was positive for HPV 31/33/35. HPV was not detected in any of the benign lesions. 41 cases were negative for both HPV DNA and p16 protein. 96 cases expressed strong p16 staining out of which 73 were positive for high risk HPV. The samples positive for low risk subtypes showed sporadic p16 positivity and belonged to LSIL category. Thus a statistically significant association (p value <0.001) exists between high risk HPV and strong p16 expression (72).

The usefulness of combination of p16 expression and MIB-1 proliferation index in detecting cervical neoplasias was studied by Srivastava et al in 2013. p16 expression was absent in all benign lesions and MIB-1 proliferation was restricted to basal one third. Strong p16 expression was detected in 8/10 LSIL/CIN 1 cases, 5/5 HSIL/CIN 2 cases, 3/3 HSIL/CIN 3 and 15/15 squamous cell carcinoma cases. Increased MIB-1 proliferation index was detected in 14 out of 15 cases of CIN 1 and all the cases of CIN 2, CIN 3 and invasive carcinomas. Thus it was concluded that with increasing severity of cervical dysplasia, there was consistently strong staining of p16 and increasing MIB-1 proliferation index (73).

Once the use of p16 as a diagnostic marker was indisputably established, its role in prognostication and management was investigated. Promising biomarkers have emerged that are associated with E6 and E7 viral proteins. A study done by Pacchiarotti et al, used p16 for prognostication of cases with HPV positivity or positive cytology but

absent high grade dysplasia (above CIN 2). All cases negative for malignancy and CIN 1 histology were followed up for 6 to 12 months with colposcopy or cytology. 83.5 % of the patients had at least one follow up. 30.6 % were CIN 1 cases, 67.2 % were negative for intraepithelial lesion and 2.2 % were inadequate histology. 79.4 % of all cases were negative for p16 staining, 17.3 % were positive for p16 and 3.2 % cases were invalid. During follow up 10 cases progressed to CIN 2 and 3 cases to CIN 3, out of which 6 cases were positive for p16 (sensitivity 46 %). There was a significant difference in absolute risk among p16 positive women (9.4/100) compared to p16 negative cases (1.7/100). CIN 1 cases had a higher risk compared to histologically negative patients (relative risk 5.2). Thus it was confirmed that p16 is a reliable prognostic marker for follow up after a negative colposcopy (74).

However, there was a contradictory study which stated that p16 expression was unable to predict the outcome of cervical intraepithelial neoplasia grade 2. Ninety women were diagnosed to have high risk HPV and CIN 2 out of which 45 women underwent excision and 45 women were followed up for 1 year at 3 months interval. p16 expression was analysed in all cases and 68.9 % of the cases showed positivity. 22 % showed progression to CIN 3, in 11 % of women, the lesion persisted, 42 % had spontaneous regression and the lesion was downgraded to CIN 1 in 11 %. There was no significant difference in regression between women who were p16 positive and negative (adjusted HR 1.1). Since rate of spontaneous regression was high, the authors advise follow up as a management option in compliant women(75).

The role of p16 as predictive marker in lymph node metastasis was studied by Huang et al. Of the 145 cases of invasive squamous and adenocarcinoma studied, 32.4 % showed strong expression, 42.1 % showed moderate staining and 25.5 % showed weak or

absent expression for p16. Over expression of p16 demonstrated significant association with histological cell type (Squamous > Adenocarcinoma) (p value = 0.015), FIGO staging (p value = 0.041) and lymph node metastasis (p value = 0.005). However, there was no significant association with grade, lymphovascular invasion, parametrial invasion, tumour size, depth of stromal invasion and vaginal invasion. The 5 year survival rate was 65.5 %. Kaplan Meier curve demonstrated that p16 over expression was associated with a poorer survival compared to p16 underexpression (59.3% vs. 83.8%). The mean survival of women with p16 under expression was 171.6 months and that of women with p16 over expression was 130.0 months. In addition to p16 expression, other factors associated with poor survival were advance FIGO stage, tumour size, vaginal involvement, lymphovascular and parametrial invasion and nodal metastasis (6).

The presence of p16 negative high grade cervical lesions and carcinomas was pointed out by Volgareva et al. 62.7 %, 68.4 %, 33.3 % and 3.8 % of CIN 1, CIN 2, CIN 3 and invasive carcinomas were negative for p16. 10 % of cases with only koilocytic change showed p16 positivity. The authors suggested that epigenetic mechanisms like loss of heterozygosity and promoter hypermethylation that cause silencing of p16 is the probable cause for absent or reduced expression of p16 (76).

As a small subset of cervical cancers is negative for high risk HPV, studies were done to differentiate the genetic profile of HPV positive and HPV negative cervical cancers. It was observed that HPV positive cervical cancers over express cyclin E, cyclin B and p16 and under express cyclin D1. Down regulation of p16 in HPV negative cancers correlated with p16 promoter hypermethylation and cyclin D1 repression linked with gene amplification (77).

After establishing that p16 can be used for diagnostic and prognostic purpose, various studies were done using a combination of several markers so as to develop a reliable panel with a sufficiently high sensitivity and specificity, which can establish cervical primary lesion and has a good interobserver agreement. The immunocytochemical staining pattern of a panel of markers comprising p16, PTEN, p53, FAS and HPV capsid protein on Thin Prep smears were studied. A total of 92 cases were evaluated. p53 was detected in 36.4 % of HSIL cases and were absent in normal and LSIL cases. PTEN positivity was observed in 97.8 % of all cases and 81.8 % of HSIL. Fas was expressed by 96.7 % of all cases and 72.7 % of HSIL. Positive expression of L1 protein and p16 was observed in 26.3 % of LSIL and 9.1 % of HSIL cases. p16 positivity with negative L1 was present in 18 % of LSIL and 81.8 % of HSIL. p16 negativity and L1 positivity was found in 5 % of normal cases, 42.6 % of LSIL, negativity for p16 and L1 was observed in 9.1 % of HSIL, 13.1 % of LSIL and 95 % of normal cases. It was found that p16 positive/L1 negative and p16 negative /L1 negative had significant association with HSIL and LSIL respectively (p value <0.001). None of the other markers had significant prognostic value in LSIL cases. Thus the authors suggest that loss of Fas and PTEN along with over expression of p53 may be used in detecting high grade lesions (78).

Another study was performed by combining p16, MIB-1 and HPV L1 capsid protein on cell blocks of residual liquid based cytology specimens. A total of 42 specimens were studied with follow up. There was 90.5 % concordance between cytology and histology. There was statistically significant difference between the expression patterns with increasing severity of dysplasia. The rate of positive staining of p16 in normal, CIN 1, CIN 2/3 and carcinoma were 0 %, 40 %, 100 % and 100 % respectively. MIB- 1 also showed a similar staining pattern of 0 %, 40 %, 87 % and 100 %. Since many of low grade lesions undergo spontaneous regression, attempts were made to predict which of

the cases are likely to upgrade into a more severe lesion. This study also demonstrated that those cases with both p16 positivity and L1 capsid positivity are likely to undergo neoplastic transformation (79).

It is known that CDKN2 encodes for p16 which is dependent on retinoblastoma pathway and p14 ARF blocks MDM2 mediated degradation of p53. 65 samples were analysed using a panel of markers, p16, p53, p14 AFR and MDM2. Over expression of MDM2 was detected in 75 % of condylomas and 16 % of dysplasias, thus making it a useful discriminatory marker between benign and malignant conditions. All the cases including carcinomas and reactive cases showed a weak positivity for p53, thus it was concluded that it was not useful in discriminating reactive and malignant conditions. p14 ARF was over expressed in all carcinoma cases, even in HPV negative carcinomas and were negative in all benign lesions. p16 was over expressed in all cases of carcinomas and there was a statistically significant difference in expression between various grades of dysplasia (80).

11850 cervical biopsy samples were analysed by 23 pathologists in a study done by Singh et al. An average of 518 cases was seen by a pathologist over 5 years. 12 pathologists were “low users” and 11 pathologists were “high users” of p16 immunohistochemical stain. When both the sets were compared, it was found that “high users” had a lower rate of discrepancies between cytology and histology (12.6 % vs. 14.92 %), greater rate of diagnosis of CIN 2 (19.9% vs. 16.4 %) and lower CIN 1/ CIN 2 ratio. (1.2 vs. 2.3) (81).

Another panel of markers, excluding p16 were used; MIB-1, c-erb2 and cyclin D were used in CIN 1, CIN 2, CIN 3 and squamous cell carcinomas. High risk HPV was detected in 82% of LSIL, 89 % of HSIL and 100 % of carcinomas. 9%, 33 % and 50 % of LSIL, HSIL

and carcinomas showed positivity for c-erb2. Nuclear expression of cyclin D1 was present in 82 %, 11 % and 30 % of CIN1, CIN 2 and squamous cell carcinoma cases respectively. It was observed that cytoplasmic cyclin D1 expression increased with increasing severity of lesion. The presence of both nuclear and cytoplasmic cyclin D1 positivity was related to high risk HPV infection (82).

Evaluation of expression pattern of p16 and MIB-1 was done in cervical cone biopsies. Diffuse expression of p16 was detected in 57.1 % of CIN 1, 73.9 % of CIN 2 cases and 100 % of CIN 3 and carcinoma cases. MIB-1 was coexpressed in the same cells that showed strong and diffuse positivity for p16. Thus, the study reflects that p16 is a marker for proliferation competent cells (83).

When ProExC was combined with p16 for detecting cervical dysplastic lesions, it was found that p16 had a superior sensitivity for CIN 2 and CIN 3 lesions (79% and 90%) compared to ProExC which had a sensitivity of 67 % and 84 % respectively for CIN 2 and CIN 3. However, in terms of specificity ProExC was superior with 100 % specificity for CIN 2 and 93 % for CIN 3. But taking into account cost effectiveness, p16 had a better overall performance as a marker for dysplasia than ProExC (84).

Another study included cyclin E to the panel of p16 and MIB-1. Histological diagnosis had a significant correlation with all the three markers (p value <0.01). MIB-1, Cyclin E and p16 had positive scores of 94.7%, 91.6% and 100 % respectively in cases of HSIL and 68.4 %, 96.7% and 100 % in cases of LSIL. Cyclin E and p16 were equally sensitive to detect high grade and low grade intraepithelial lesions. Reactive epithelial cells had a score of 7.7 %, 8.0 % and 12 % respectively for the three markers (85).

Histologically, adenocarcinoma poses a greater diagnostic challenge and usually requires a panel of markers. p21 expression along with p16, cyclin E and p27 were

analysed. There was significant correlation between the expression of p16 and p27, cyclin E and p27, and cyclin E and p21. Negative staining for p21 was also associated with an overall poor survival (86).

A study done by Cameron et al used a panel of p16, MIB-1 and bcl-2 to differentiate between glandular neoplasia and benign conditions like endometriosis, endometrial metaplasia and microglandular hyperplasia. Cases of glandular neoplasia showed strong positivity for p16 with high MIB-1 proliferation index and a negative staining for bcl-2. Endometriosis and endometrial metaplasia showed strong bcl-2 positivity, focal p16 positivity and low MIB-1 proliferation index. Microglandular hyperplasia demonstrated negativity for bcl-2 and p16 along with low MIB-1. Thus, the panel was successful in discriminating the reactive and neoplastic lesions(87).

Role of p16 has been investigated in other tumours and was observed that high grade serous ovarian carcinomas had a significantly stronger expression of p16 compared to low grade and borderline tumours. (88). The role of p16 and association of HPV have been extensively studied in orpharyngeal carcinomas and p16 emerged as the strongest independent predictor of prognosis, even more than tumour size, stage and nodal status(89).

To conclude, various studies have proven that p16 is a reliable surrogate marker for cervical dysplasia in terms of sensitivity, specificity, discriminatory ability and cost effectiveness. p16 has also been used as a prognostic marker to determine overall survival and disease free survival.

AIM

To study the p16 expression pattern in premalignant and malignant lesions of cervix in biopsy specimens.

OBJECTIVES

a. To evaluate the diagnostic utility of p16 to differentiate between the premalignant and malignant squamous and glandular lesions of cervix.
b. To assess the utility of p16 to distinguish between benign and dysplastic lesions of cervix.
c. To assess the clinical impact of p16 expression on the disease outcome.
d. To assess the performance of cytology as a screening tool compared to histology.

MATERIALS AND METHODS

All the procedures involved in the study were approved by the Institutional Review Board of Christian Medical College, Vellore. Cervical biopsy samples received in the department of General Pathology, Christian Medical College, Vellore, from 1st June 2011 to 31st May 2013 were utilised for the study. The study was retrospective and archival stained and mounted slides and formalin fixed paraffin embedded tissue blocks were retrieved. The samples had been fixed overnight in 10% buffered formalin and were embedded in paraffin wax using conventional methods. Haematoxylin and eosin stained slides of all cases of cervical biopsy were reviewed.

Strict histomorphological criteria were used to classify all the cases into four distinct categories:

1st category – All reactive non dysplastic conditions which mainly include infections, inflammation, regenerative changes, hyperplasia, endometriosis, post radiation changes endocervical polyps, koilocytosis and metaplasias.

2nd category – Different levels of dysplasia, i.e., premalignant lesions of cervix, Cervical Intraepithelial Neoplasia, grades 1, 2 and 3(CIN 1, 2 and 3).

3rd category – Invasive squamous cell carcinomas.

4thcategory–Microglandular hyperplasia, adenocarcinoma in situ and invasive adenocarcinoma.

Clinical and radiological data were obtained from clinical workstation and the initial pathological diagnosis was reviewed from Pathology workstation using PACS (Picture Archival and Communication System).

The clinical parameters evaluated were age at the time of diagnosis, mode of presentation, relevant history, cytological diagnosis, details of other related procedures like colposcopy and presence of iodine acetowhite areas, appearance of cervix during clinical examination, evaluation of uterus and adnexal structures. In case of malignancies, radiological staging, lymph node metastasis, invasion into pelvic structures, and distant metastasis were also noted. Previous biopsies and follow up biopsies and Pap smears were also evaluated to assess disease progression.

CRITERIA FOR DIAGNOSIS

Criteria for diagnosis for CIN

Nuclear abnormalities

(1) Increased nucleo-cytoplasmic ratio due to disproportionate nuclear enlargement

(2) Presence of hyperchromasia and irregular clumping of chromatin

(3) Anisokaryosis and pleomorphism of nuclei

(4) Loss of nuclear polarity

(5) Irregular nuclear membrane.

Nuclear atypia of premalignant lesions is distinguished from the nuclear atypia of reactive changes by the heterogeneity of nuclear changes in premalignant conditions, in contrast to relatively homogenous changes in atypia of reactive conditions. Nucleoli are relatively rare in premalignant conditions.

Mitotic activity

(1) Number of mitotic figures was counted in 10 high power fields (40 X magnification).

(2) Location of mitotic figures – lower, middle or upper one third of the epithelium.

(3) Presence of abnormal configurations.

It is described as

Lag type mitosis - non attached chromatin in metaphase in the area of mitotic figure.

Three group metaphase – the main bulk of chromatin aligns along the equator and non attached chromatin lies laterally at the two polar sites.

Two group metaphase – displaced chromatin is present only in one pole.

Other less common forms are multipolar tripolar and bizarre mitotic figures.

Differentiation

Proportion of epithelium showing differentiation – basal one third, two third or full thickness dysplasia.

Criteria for diagnosis of squamous cell carcinoma

Nuclear and cytological abnormalities mentioned above along with

(1) Stromal invasion; extent of invasion – less than 3mm invasion into stroma is termed microinvasive carcinoma. Any macroscopically visible tumour or tumour with more than 3mm of stromal invasion is considered invasive carcinoma.

(2) Presence of stromal desmoplasia and necrosis

(3) Grade of differentiation –

Well differentiated (grade 1): mature squamous cells with the presence of abundant keratin including formation of keratin pearls and intercellular bridges.

Moderately differentiated (grade 2): Less abundant keratin with focal individual cell keratinisation, less well demarcated cell borders, higher mitotic activity and nuclear pleomorphism.

Poorly differentiated (grade3): Sheets and nests of primitive cells with marked pleomorphism and high nuclear cytoplasmic ratio, presence of basaloid and spindle cell morphology or squamous differentiation made out only on immunohistochemistry.

Criteria for diagnosis glandular neoplasia

(1) Presence of intracellular mucin

(2) Increased nucleo- cytoplasmic ratio

(3) Mitoses evaluated as above

(4) Apoptosis

(5) Nuclear pleomorphism

(6) Nuclear hyperchromasia

(7) Loss of nuclear polarity.

Criteria for diagnosis of invasive adenocarcinoma

Above mentioned criteria with stromal invasion along with stromal desmoplasia or necrosis.

Grade of differentiation – Well differentiated adenocarcinoma (grade 1): Presence of well formed glandular structures with easily demonstrable Periodic Acid Schiff positive mucin and less than 5% solid areas.

Moderately differentiated adenocarcinoma (grade 2): Less conspicuous glandular architecture with less amounts of mucin and 5 to 50% solid areas.

Poorly differentiated adenocarcinoma (grade 3): Solid sheets of tumour amounting to more than 50% with occasional pseudo rosettes and palisaded nuclei and little intracytoplasmic mucin.

Endocervical origin of the tumours was confirmed by positive immunohistochemical expression for CEA and negative expression of Vimentin and ER.

Criteria for diagnosis of reactive changes

(1) Absence of features of CIN, glandular neoplasia or invasive malignancy.

(2) Predominance of inflammatory component.

Criteria for diagnosis of koilocytes

(1) Sharply defined perinuclear halo.

(2) Peripheral condensation of cytoplasm

(3) Nuclear size two to three times that of an intermediate cell or multinucleation

(4) Wrinkling of nuclear membrane.

A total of 284 cervical biopsy specimens were received, out of which 53 were excluded due to inadequate tissue, presence of erroneous tissue (mainly endometrial samples), non availability of paraffin embedded blocks or scant tissue remaining in the block inadequate for immunohistochemistry, other morphological variants of cervical carcinoma and slide and block review. The remaining 231 cases were reviewed and a total of 120 cases which strictly obeyed the diagnostic criteria mentioned above for various categories were included in the study. 15 cases each of CIN 1, 2 and 3, 15 cases of invasive squamous cell carcinoma and 22 cases of adenocarcinoma and 38 cases of reactive condition were chosen. The relatively higher number of preinvasive lesions

was included as they pose greater diagnostic challenge compared to frank invasive squamous cell carcinoma.

Histology

At least three H&E (Haematoxylin and eosin) stained sections of all 120 cases were reviewed by two pathologists. A consensus was reached and only the cases that strictly adhered to the diagnostic criteria were selected for the study. A CH-20i microscope (Olympus Corporation, Japan) was used for the study.

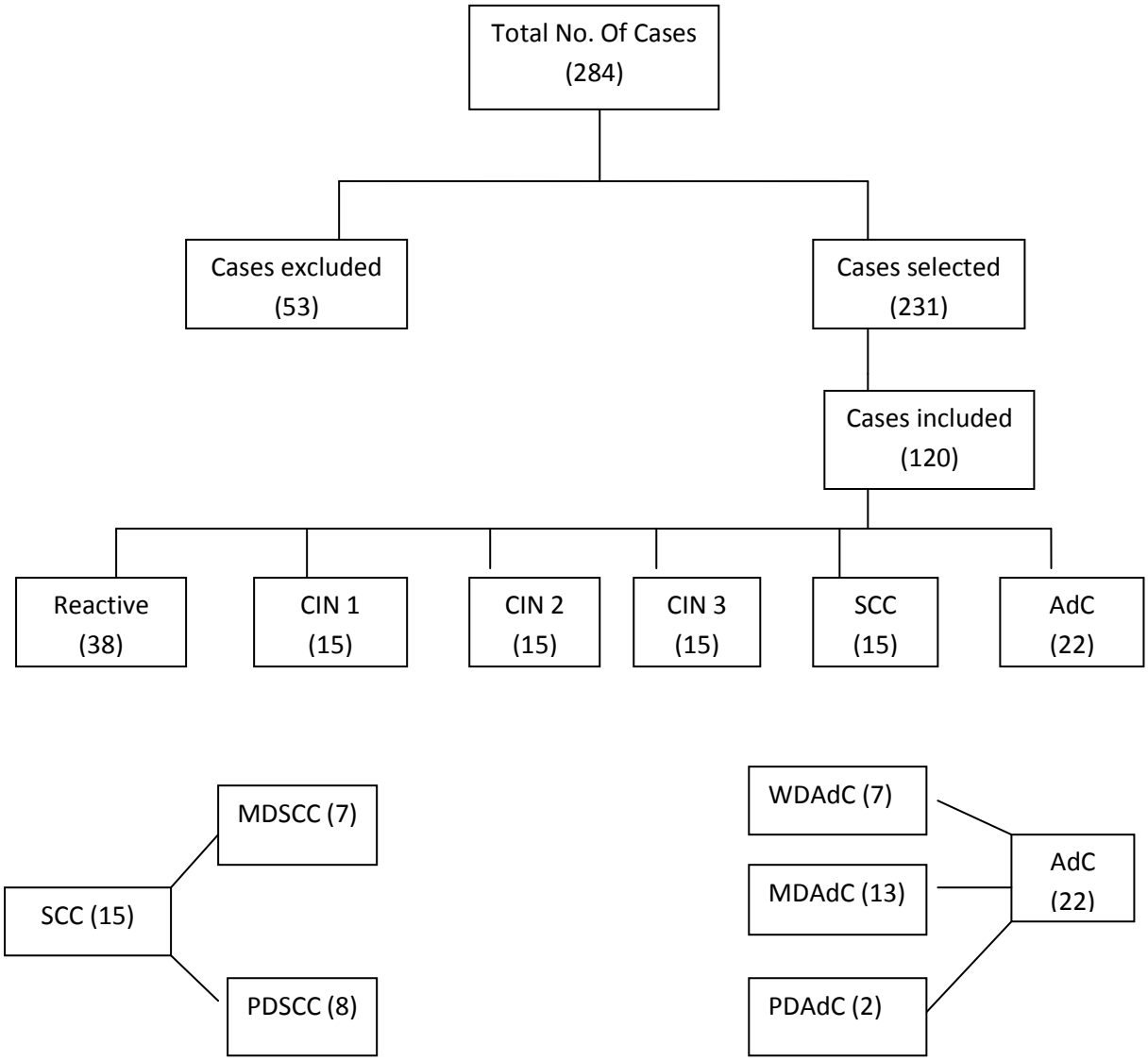

Figure 4: Flow chart depicting the distribution of cases included in the study.

p16 INK4A immunohistochemistry was performed on 120 tissue biopsies. The antibody specifications are as follows:

Manufacturer: CINtech, Ventana Medical Systems.

Species: Mouse monoclonal

Isotype: IgG2a

Clone name: E6H4

Catalogue number: 725 – 4713.

Steps for immunohistochemistry

(1)Tissue was fixed overnight in 10% buffered formalin.

(2)Paraffin embedded tissue sections were cut at 5 micrometer thickness and floated onto poly L – lysine coated slides

(3)Overnight incubation at 37 degree Celsius.

(4)The slides were treated with 4% milk solution for 10 minutes to eliminate the hydrophobic effect and to give positive charge to the slides.

(5)Slides were then bar-coded

(6)Endogenous peroxidise blocking using 0.3% hydrogen peroxide for 10 minutes.

(7)Immunohistochemistry was performed on the labelled slides using Ventana Benchmark XT autostainer (fully automated stainer).

(8)Heat mediated antigen retrieval was done by treating with standard cell conditioning 1 (CC1) solution (pH patent with company) for one hour at 90 degree Celsius.

(9)Addition of primary antibody and incubation for 40 minutes at 37 degree Celsius.

(10) Wash with reaction buffer

(11) Addition of secondary antibody (multimer) and incubation for 8 minutes.

(12) Wash with reaction buffer

(13) Counter stain with Haematoxylin for 8 minutes.

(14) Incubate with bluing agent for 4 minutes.

(15) The slides were then brought to 80% alcohol (2 changes) to remove the liquid cover slip followed by drying and mounting in DPX.

(16) Counter stain with copper haematoxylin (10%) and blue ink.

A tissue section of invasive squamous cell carcinoma of cervix was taken as external positive control in each batch.

Immunopositivity for p16 was evaluated using three parameters:

A semi quantitative scoring system was done based on intensity and proportion of staining.

Table 1: Scoring system for p16 used in this study

INTENSITY	PROPORTION	PATTERN
0 - No staining 1 – Weak staining 2 – Moderate staining 3 –Strong staining	0 – No staining 1 – Staining in < 1% of cells 2 – Staining in 1 – 10 % of cells 3 – Staining in 11 – 33 % of cells 4 – Staining in 34 – 66 % of cells 5 – Staining in > 66 % of cells	N – Nuclear C – Cytoplasmic M - Membranous

Statistical Analysis

Data was analysed using Statistical Package for Social Sciences (SPSS) software (Windows version 16). Descriptive statistics for continuous data was expressed as mean with S.D or median. Categorical data was expressed as frequencies and percentages. Sensitivity and specificity of p16 expression in cervical dysplasia were obtained from ROC curves. Association between koilocytosis and CIN was calculated using Fisher's exact chi square test. Association between p16 expression and radiological parameters and tumour differentiation was calculated using non parametric Kruskal Wallis test. Correlation of intensity and proportion of p16 expression with severity of lesion was obtained from Bland Altman plot. Time taken for progression of lesion was derived from Kaplan Meir curve. For all statistical analysis, p value < 0.05 was considered significant.

RESULTS

A total of 120 cervical biopsy samples were studied - 15 cases each of CIN 1, CIN 2, CIN 3 and squamous cell carcinoma out of which 8 were poorly differentiated and 7 were moderately differentiated tumours. Of the remaining 60 cases, 38 were reactive and benign lesions of squamous and glandular compartments and 22 cases were adenocarcinoma out of which 7 were well differentiated, 13 moderately differentiated and 2 were poorly differentiated tumours.

Table 2: Distribution of cases according to the diagnosis included in the study

Cervical Intraepithelial Neoplasia	Total No: of cases – 45
CIN 1	15 (12.5%)
CIN 2	15 (12.5%)
CIN 3	15 (12.5%)
Squamous cell carcinoma	Total No: of cases – 15
Moderately differentiated	7 (5.9%)
Poorly differentiated	8 (6.7%)
Adenocarcinoma	Total No: of cases – 22
Well differentiated	7 (5.9%)
Moderately differentiated	13 (10.9%)
Poorly differentiated	2 (1.2%)
Reactive and benign conditions	Total No: of cases – 38
Squamous lesions	28 (23.4%)
Glandular lesions	10 (8.3%)

Reactive lesions included chronic cervicitis (15 cases), regenerative atypia (2 cases), squamous metaplasia (2 cases), endometriosis (1 case), granulomatous inflammation (1 case) and koilocytosis (6 cases), microglandular hyperplasia (6 cases), papillary endocervicitis (2 cases) and endocervical polyp (2 cases).

The mean age of patients was 45.5 years with a range 18 to 70 years. The mean age of patients with CIN 1 was 43.3 years (Range 27 – 63 years), CIN 2 was 41.4 years (Range 23 – 60 years), CIN 3 was 48.6 years (Range 35 – 61 years), squamous cell carcinoma was 52.0 years (Range 42 – 68 years) and adenocarcinoma was 50.0 years (Range 36 – 70 years).

54.05 % of patients with cervical carcinomas were from Tamil Nadu (54.05 %) followed by West Bengal (29.32 %), Andhra Pradesh (8.10 %), Jharkhand (5.4 %) and north eastern states (2.7 %).

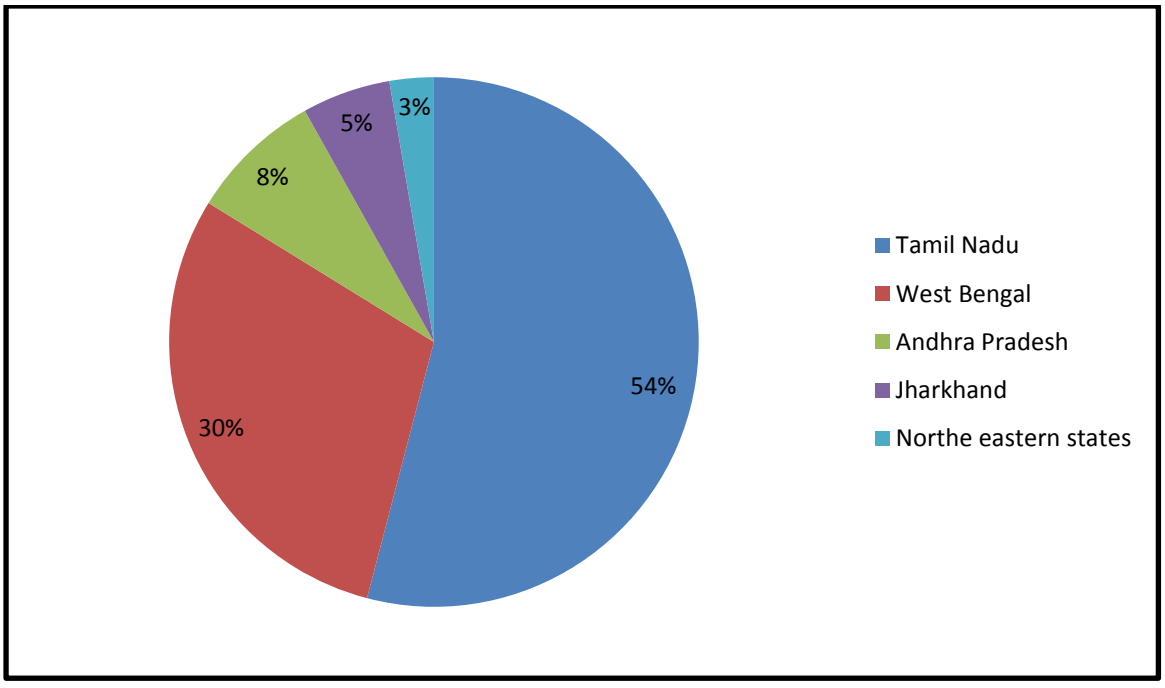

Figure 5: Regional distribution of cervical cancer patients

The most common presenting symptom was abnormal vaginal discharge (30.8 %) followed by cervical growth (25.0 %), abnormal uterine bleeding (21.7 %), cytological abnormality in Pap smear detected during routine cervical screening (15.8 %) and post coital bleeding (6.7 %).

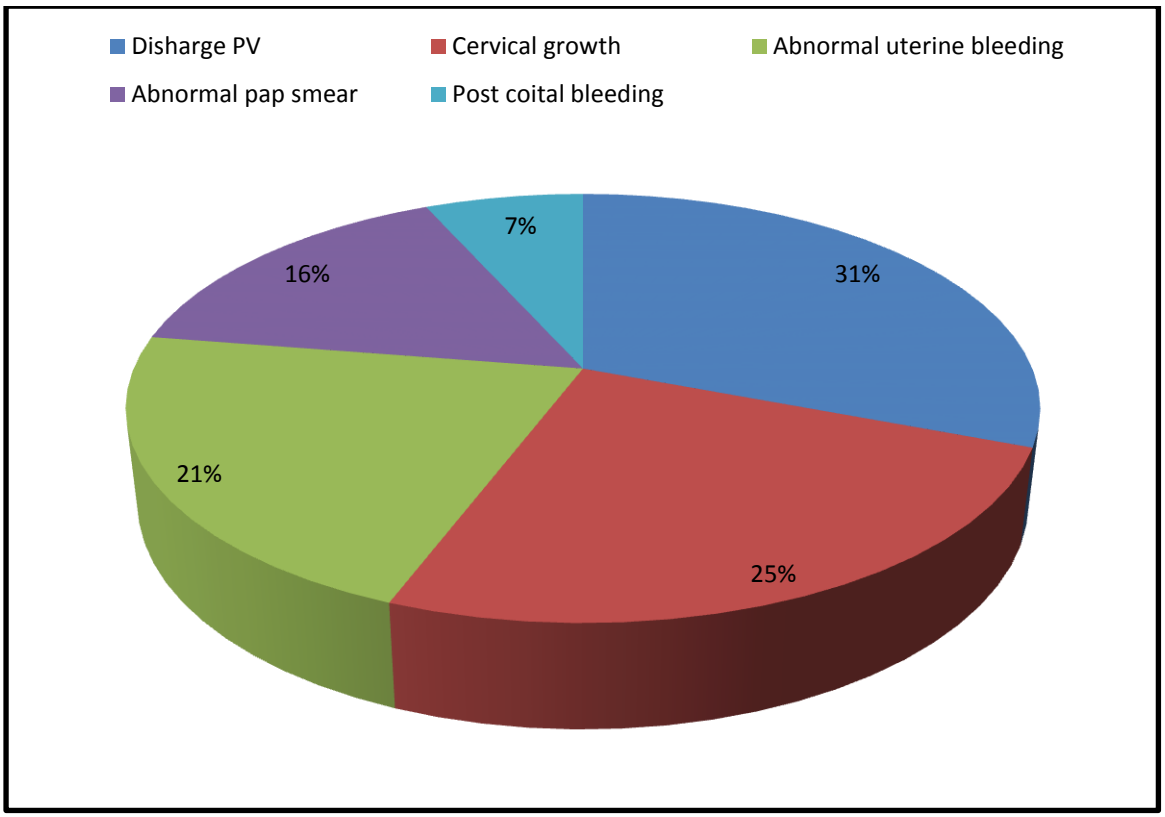

Figure 6: Distribution of cases according to mode of presentation.

83.3 % of total number of patients underwent punch biopsy. The other types of biopsies performed were LEEP (Loop Electrosurgical Excision Procedure) and LLETZ (Large Loop Excision of Transformation Zone) which were restricted to CIN cases. Out of 15 CIN 1 cases, 7 patients had under gone LEEP and 3 patients had undergone LLETZ. Out of 15 CIN 2 cases, 2 patients each had undergone LEEP and LLETZ and in 15 CIN 3 cases, 1 patient and 4 patients had undergone LEEP and LLETZ respectively.

Table 3: Distribution of CIN cases according to the type of biopsy.

Diagnosis	Punch biopsy	LEEP	LLETZ
CIN 1	5 (33.3 %)	7 (46.6 %)	3 (20 %)
CIN 2	11 (73.3 %)	2 (13.3 %)	2 (13.3 %)
CIN 3	10 (66.6 %)	1 (6.6 %)	4 (26.6 %)

Koilocytic change was seen in 28 out of 120 cases. 8 cases of CIN 1 (53.3 %), 10 cases of CIN 2 (66.6 %) and 4 cases of CIN 3 (26.6 %) showed koilocytic change in the adjacent epithelium. There was a statistically significant association between koilocytic change and CIN 1 and CIN 2 (p value 0.03 and < 0.01 respectively) and not with CIN 3 (p value = 0.7). However, it was observed that there was no statistically significant difference within each subtype of CIN and koilocytic change.

Table 4: Association between koilocytosis and CIN

Diagnosis	Koilocytes present	Koilocytes absent	P value
Reactive	6 (20 %)	24 (80 %)	
CIN 1	8 (53.3 %)	7 (46.7 %)	0.03
CIN 2	10 (66.6 %)	5 (33.3 %)	<0.01
CIN 3	4 (26.6 %)	11 (73.3 %)	0.7

The morphological features analysed were (1) Nuclear enlargement, hyperchromasia, membrane irregularity. (2) loss of polarity, (3) mitotic count. Out of these, it was observed that mitotic count was the only parameter which showed statistically

significant difference within the subtypes of CIN. (Calculated using Kruskal Wallis non parametric test, p value <0.001).

Table 5: Clinical features and histological parameters in the cervical dysplastic lesions.

	CIN 1	CIN 2	CIN 3	SCC	AdC
Mean age (years)	43.3	41.4	48.6	52.0	50.0
Commonest mode of presentation	Abnormal Pap smear	Vaginal discharge	Vaginal discharge	Cervical growth	Cervical growth
Koilocytic change	53.3 %	66.6 %	26.6 %		
Mean mitotic count/ 10hpf	4.1	5.6	9.4	11.4	10.5

P16 expression in cervix in reactive, premalignant and malignant squamous and glandular lesions.

Amongst the reactive lesions, 2 cases of squamous metaplasia, 2 cases of chronic cervicitis, 1 case of microglandular hyperplasia and 1 case of endometriosis showed patchy weak cytoplasmic positivity for p16. The remaining 34 cases including the 6 cases of koilocytosis were completely negative for p16.

Table 6: Pattern of p16 expression in various cervical biopsy specimens

	Total no: of cases positive for p16	Strong	Moderate	Weak
CIN 1	11/15(73.3 %)	3/11(27.2 %)	4/11(36.3 %)	4/11(36.3 %)
CIN 2	14/15(93.3 %)	9/14(64.2 %)	5/14 (35.7 %)	
CIN 3	14/15 (93.3 %)	14/14(100.0%)		
SCC	15/15(100.0 %)	14/15(93.3 %)	1/15(6.6 %)	
AdC	21/22(95.4 %)	18/21(85.7 %)	3/21(14.2 %)	
Reactive	6/38(15.7 %)			6/38 (15.7 %)

Expression pattern of p16 in CIN 1

11 out of 15 cases of CIN 1 showed nuclear positivity for p16, out of which 4 cases demonstrated weak staining, 4 cases moderate staining and 3 cases showed strong staining. 4 cases showed complete absence of p16 immunostaining. ROC curve (Receiver Operator Characteristic) was generated to assess the performance indicators. When the threshold was set at a score of 2, the sensitivity, specificity, positive predictive value and negative predictive were detected to be 73.3 %, 85.7 %, 73.3 % and 85.7 %. With the cut off set at a score of 5, the sensitivity dropped to 26.6 %. The positive predictive value and negative predictive value were 50.0 % and 68.5 % respectively.

Table 7: Performance indicators of p16 in CIN 1

	Sensitivity	Specificity	PPV	NPV
Score 2	73.3 %	85.7 %	73.3 %	85.7 %
Score 5	26.6 %	85.7 %	50.0 %	68.5 %

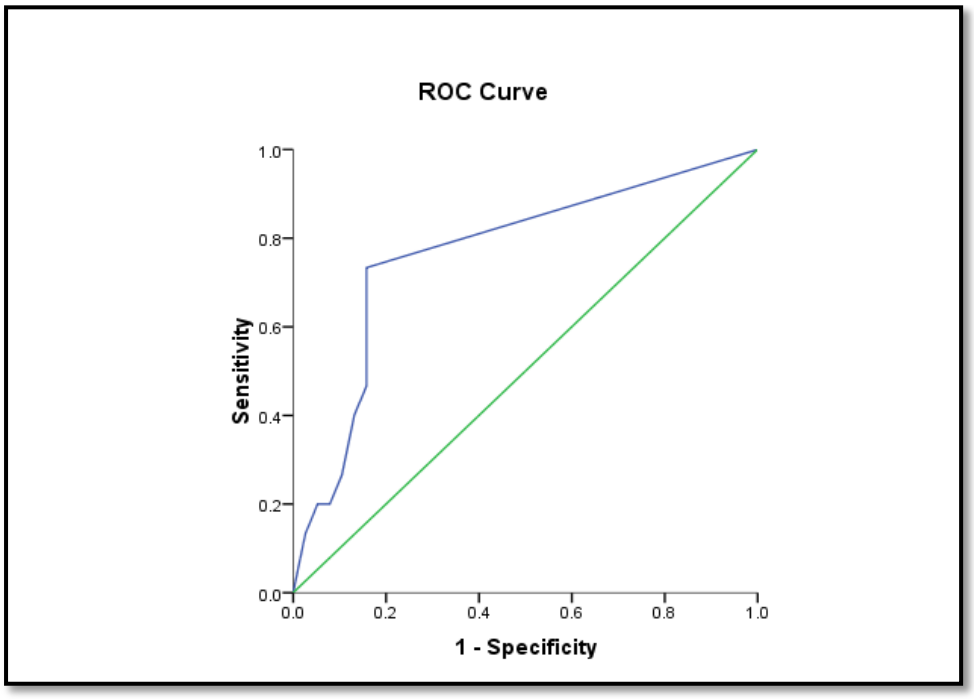

Figure 7: ROC curve depicting p16 expression in CIN 1

Expression pattern of p16 in CIN 2

Out of 15 cases of CIN 2, 14 cases showed positivity for p16 out of which strong and diffuse nuclear positivity was observed in 9 cases (64.2 %) and moderate positivity in 5 cases (35.7%). With the cut off set at a score of 2, the sensitivity, specificity, positive predictive value and negative predictive value obtained were 93.3 %, 85.7 %, 77.78 % and 96 % and with a cut off set at a score of 5, the sensitivity, positive and negative predictive values were found to be 86.6 %, 85.7 %, 76.4 % and 92.3 %.

Table 8: Performance indicators of p16 in CIN 2

	Sensitivity	Specificity	PPV	NPV
Score 2	93.3 %	85.7 %	77.7 %	96.0 %
Score 5	86.6 %	85.7 %	76.4 %	92.3 %

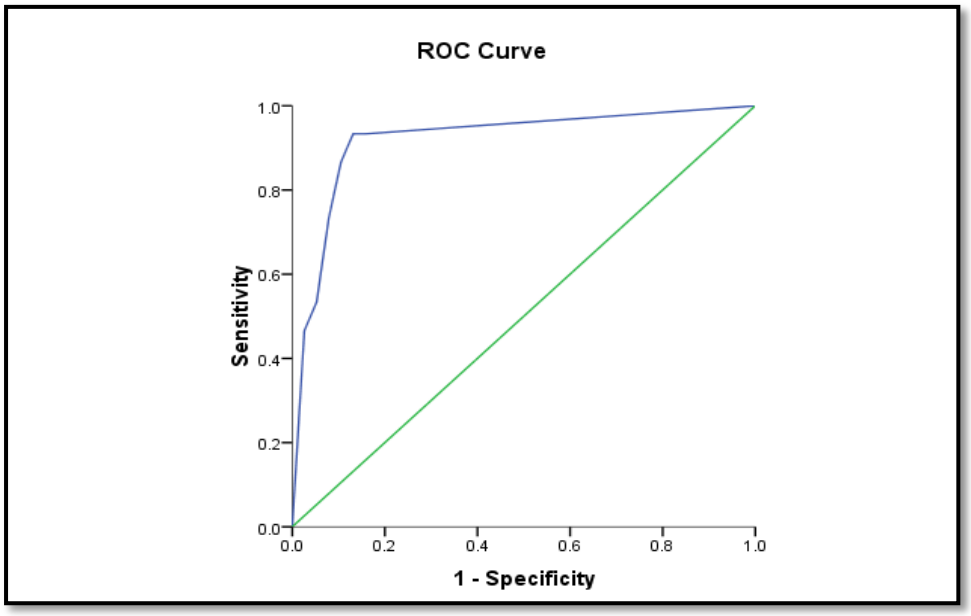

Figure 8: ROC curve depicting p16 expression in CIN 2

Expression pattern of p16 in CIN 3

Out of the 15 cases of CIN 3, 14 cases showed strong and diffuse nuclear positivity for p16 and 1 case showed complete absence of staining. With the cut off set at a score of 2, the sensitivity, specificity, positive predictive value and negative predictive value obtained were 93.3 %, 85.7 %, 77.78 % and 96 %.

Table 9: Performance indicators of p16 in CIN 3

	Sensitivity	Specificity	PPV	NPV
Score 2	93.3 %	85.7 %	77.7 %	96.0 %
Score 5	86.6 %	85.7 %	76.4 %	92.3 %

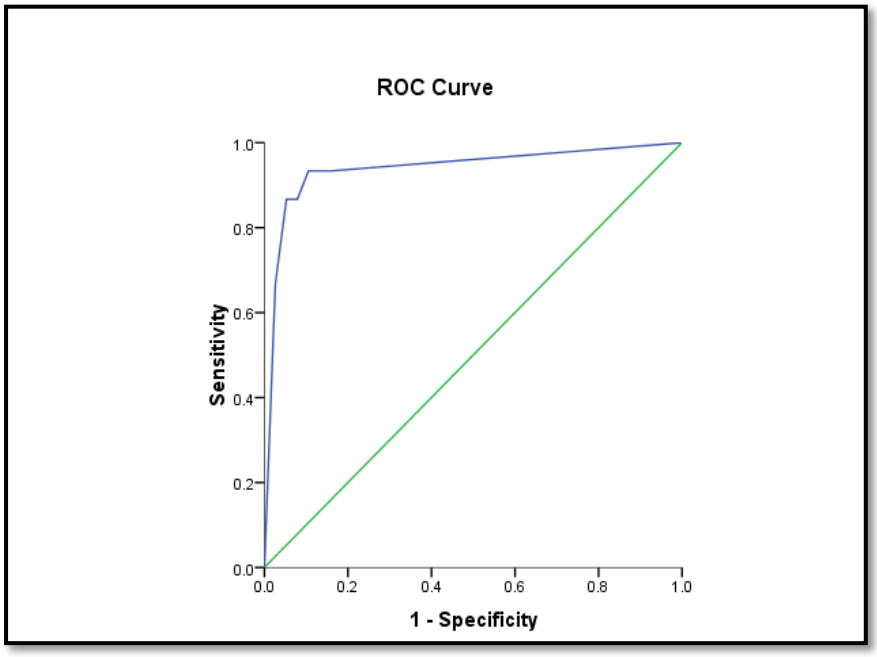

Figure 9: ROC curve depicting p16 expression in CIN 3

Expression pattern of p16 in Squamous Cell Carcinoma

Out of the 15 cases of Squamous Cell Carcinoma, all 7 cases of moderately differentiated squamous cell carcinomas and 7 out of 8 cases of poorly differentiated carcinomas showed diffuse and strong staining for p16. 1 case of poorly differentiated carcinoma showed moderate staining. With cut off set at a score of 2 and 5, the sensitivity, specificity, positive predictive value and negative predictive value observed were 100.0 %, 85.7 %, 78.9 % and 100 .0 % respectively.

Table 10: Performance indicators of p16 in SCC

	Sensitivity	Specificity	PPV	NPV
Score 2/5	100.0%	85.7 %	78.9%	100.0%

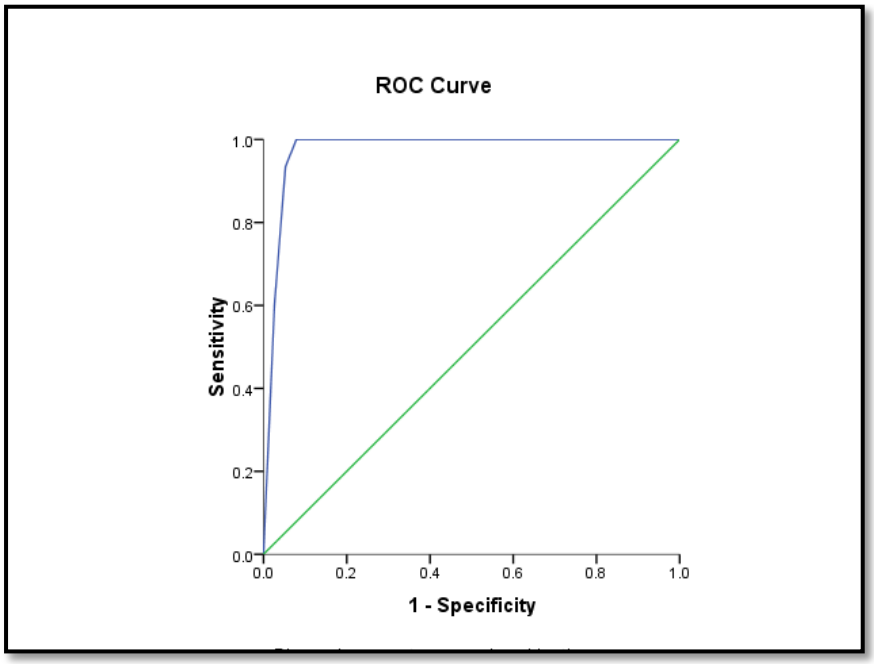

Figure 10: ROC curve depicting p16 expression in squamous cell carcinoma

Expression pattern of p16 in adenocarcinoma

Out of 22 cases of adenocarcinoma, 1 case was negative, 3 cases showed moderate staining and 18 cases showed strong and diffuse staining for p16. ROC curve generated with a cut off set and a score of 2 and 5 gave a sensitivity, specificity, positive predictive value and negative predictive value of 95.45 %, 80.0 %, 91.3 % and 88.9 % respectively.

Table 11: Performance indicators of p16 in Adenocarcinoma

	Sensitivity	Specificity	PPV	NPV
Score 2/5	95.45%	80.0%	91.3%	88.9%

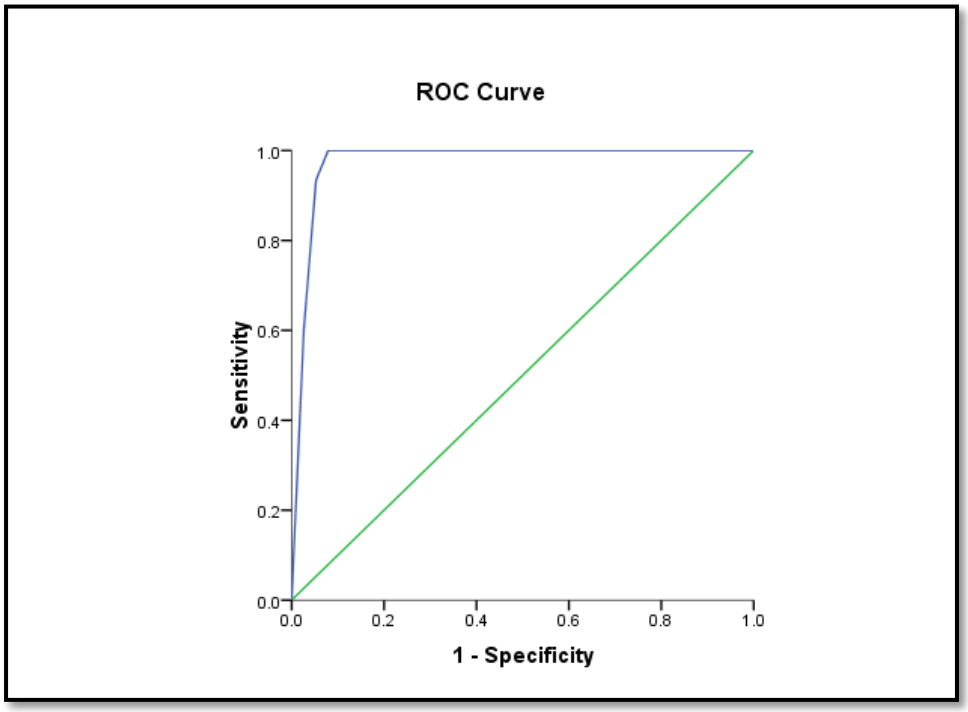

Figure 11: ROC curve depicting p16 expression in adenocarcinoma

While evaluating p16 expression, between intensity and proportion, the reaction intensity correlated better with the severity of the lesion calculated from Bland Altman plot.

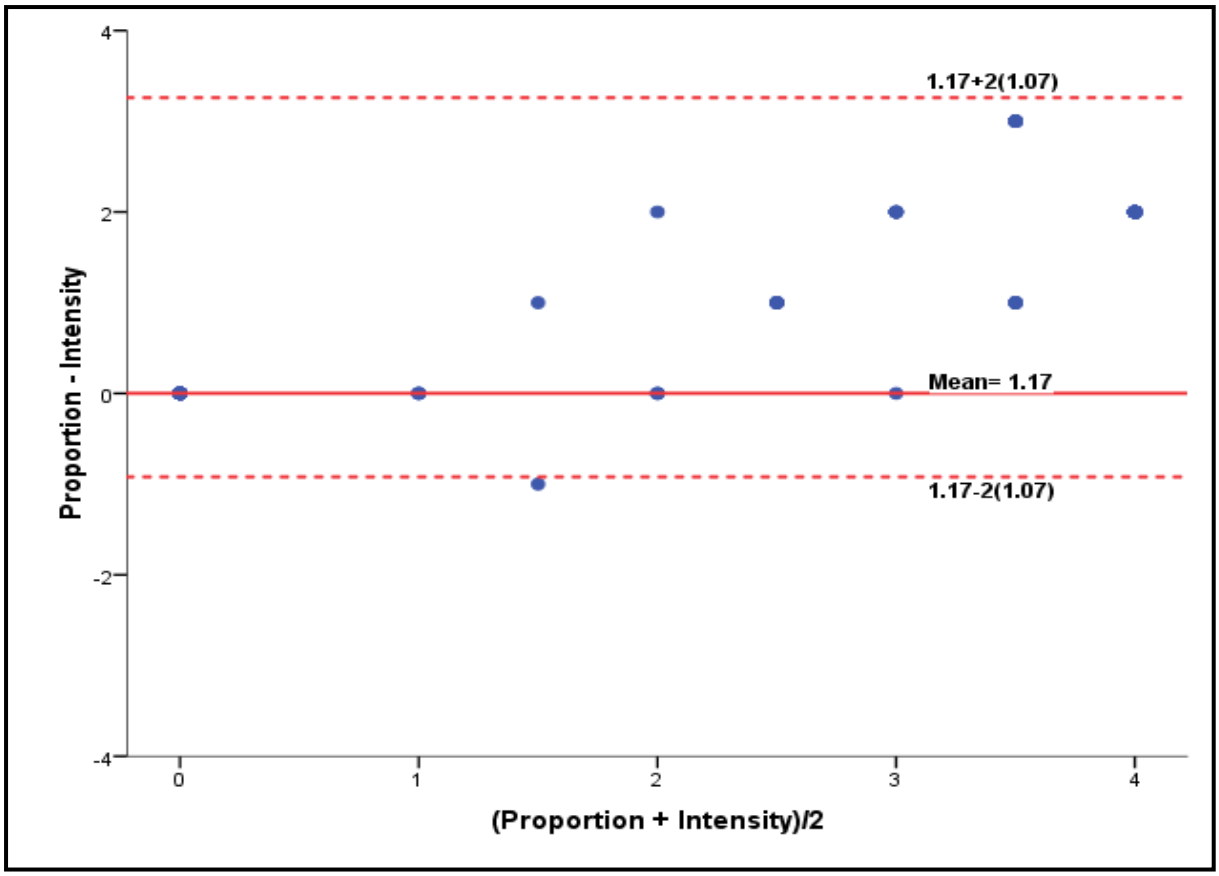

Figure 12: Bland Altman plot comparing intensity and proportion of p16 expression

Correlation of p16 with tumour differentiation

Statistical significance for association of p16 expression and differentiation of tumour was assessed (using non parametric Kruskal Wallis test). A total of 37 carcinomas were included in the study, out of which 7 were moderately differentiated squamous cell carcinoma, 8 poorly differentiated squamous cell carcinoma, 7 well differentiated adenocarcinoma, 13 moderately differentiated adenocarcinoma and 2 poorly differentiated adenocarcinoma. There was no statistically significant association

between the differentiation of tumour and p16 expression (Kruskal Wallis non parametric test, p value = 0.9)

Table 12: Correlation of p16 expression with different grades of carcinoma

	No. Of cases	P16 expression	P value
Well differentiated * Adenocarcinoma	7/7 (100.0 %)	7/7 (100.0 %)	0.9
Moderately differentiated Squamous cell carcinoma Adenocarcinoma	7/20 (35.0 %) 13/20 (65.0 %)	7/7 (100.0 %) 12/13(92.3%)	
Poorly differentiated Squamous cell carcinoma Adenocarcinoma	8/10 (80.0 %) 2/10 (20.0 %)	8/8 (100.0 %) 2/2 (100.0 %)	

*There were no cases of well differentiated squamous cell carcinoma included in this study.

Correlation of p16 overexpression with radiological parameters

p16 overexpression is defined as a total score of 8.

Radiological staging

Radiological staging was available in all 37 cases of carcinomas. Non parametric Kruskal Wallis test was used for assessment of statistical significance. It was found that p16 overexpression has statistically significant association with stage of the tumour. (p value = 0.01).

Lymph node metastasis

Out of 37 cases of carcinomas, 13 cases had documented lymph node metastasis. Using Mann Whitney U test, it was found that there is a statistically significant association between p16 overexpression and lymph node metastasis. (p value = 0.01)

Local and distant metastasis

15 out of 37 cases had no local or distant metastasis. 17 cases had local metastasis to parametrium or vagina. 5 cases had metastasized to lung or liver. There was a statistically significant association between p16 expression and metastasis. (Kruskal Wallis test; p value = <0.01)

Table 13: Correlation between radiological parameters and p16 expression.

Radiological parameter	Total No: of cases	p16 overexpression	p value
Radiological staging			
Stage I	14/37(38.8%)	3/14 (21.4 %)	
Stage II	5/37(13.8%)	3/5 (60.0 %)	0.01
Stage III	13/37(33.3%)	13/13 (100.0 %)	
Stage IV	5/37(13.8%)	5/5 (100.0 %)	
Lymph node metastasis			
Present	13/37(35.1%)	13/13 (100.0 %)	0.01
Absent	24/37(64.8%)	11/24 (45.8 %)	
Metastasis			
Absent	15/37(40.5%)	3/15 (20.0%)	<0.01
Local metastasis	17/37(45.9%)	16/17 (94.1%)	
Distant metastasis	5/37(13.5%)	5/5 (100.0 %)	

Correlation between cervical cytology and biopsy

Out of 120 cases, 74 cases had a Pap smear immediately prior to cervical biopsy. 39 cases (52.7 %) were negative for intraepithelial lesion / malignancy, 9 cases (12.1 %) were diagnosed as Low grade Squamous Intraepithelial Lesion(LSIL), 15 cases (20.2 %) as High grade Intraepithelial Lesion(HSIL), ASC – H (Atypical Squamous Cells – cannot exclude HSIL) was diagnosed in 4 cases (5.4 %), Atypical Glandular Cells, favour neoplastic was diagnosed in 3 cases (4.05%) and 1 case was diagnosed as squamous cell carcinoma (1.35 %) and 3 smears (4.05 %) were unsatisfactory for evaluation.

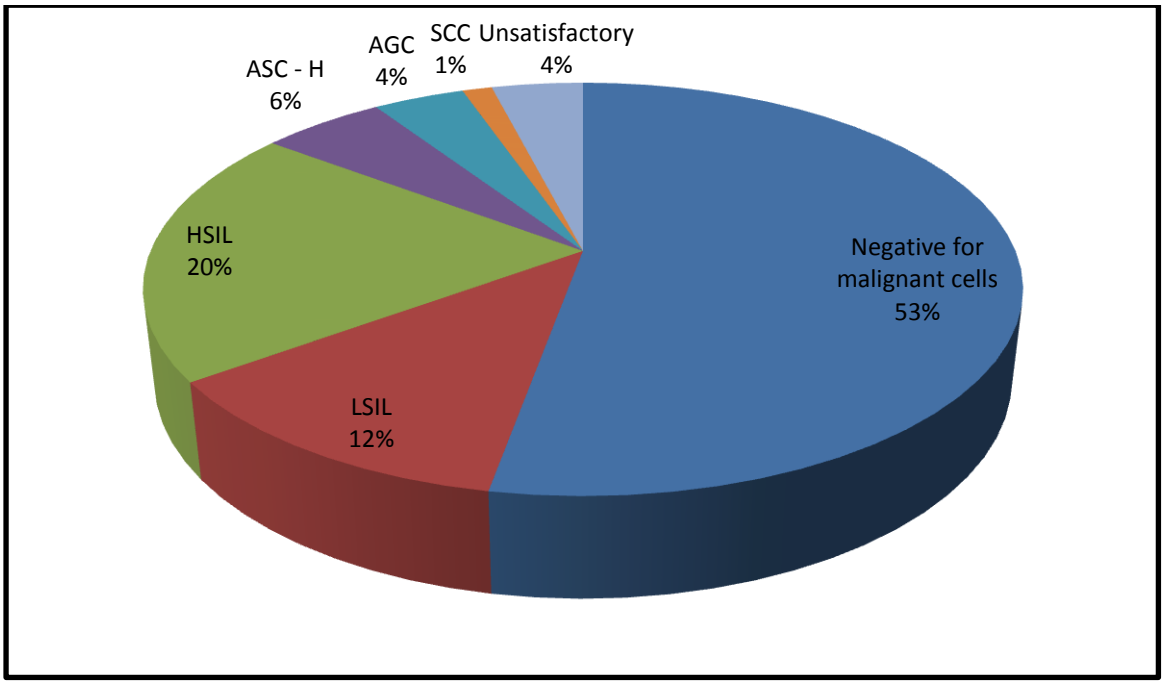

Figure 13: Distribution of cases according to diagnoses on Pap smear.

Out of all the 38 reactive conditions, 27 cases had previous Pap smear diagnosis. All the cases were reported as negative for intraepithelial lesion / malignancy.

All the 15 cases of CIN 1 had previous Pap smears out of which 7 cases were reported as negative for intraepithelial lesion / malignancy. 5 cases were reported as LSIL, 1 case as ASC – H, 1 case as atypical glandular cells and 1 smear was unsatisfactory for evaluation. Thus, the sensitivity and specificity of cytology to detect CIN 1 was found to be 41.67% and 100 % respectively. The positive predictive value was 100 % and negative predictive value was 79.4 %.

Out of 45 CIN 2/3/SCC cases, 27 cases had previous Pap smear, HSIL was diagnosed in 14 cases, LSIL in 4 cases, ASC – H in 3 cases, squamous cell carcinoma in 1 case and negative for intraepithelial lesion in 4 cases. 1 smear was unsatisfactory for evaluation. Thus, the sensitivity and specificity of HSIL to detect a high grade lesion was 65.2% and 100% respectively. The positive predictive value was 100 % and negative predictive value was 84 %.

A diagnosis of ASC – H was made in 4 cases, out of which 3 cases were diagnosed as CIN 3 on biopsy and 1 case as CIN 1.

Out of 22 cases of adenocarcinoma, only 5 patients had undergone Pap smear test. 1 smear was reported as negative for malignant cells, 2 cases as atypical glandular cells, favour neoplastic, 1 case as HSIL and 1 case as unsatisfactory for evaluation. The sensitivity and specificity of cytology in adenocarcinoma was found to be 66.67 % and specificity to be 100 %. The positive and negative predictive values were 100 % and 87.5 % respectively.

Table 14. Distribution of cytology cases according to diagnoses

	CIN 1	**CIN 2/3/SCC**	**Adenocarcinoma**	**Reactive**
Total No. Of cases	15/15 (100%)	27/45(60.0 %)	5/22 (22.7 %)	27/38(71.05%)
LSIL	5/15(33.3%)	4/27 (14.8%)		
HSIL		14/27(51.8%)	1/5 (20.0%)	
ASC-H	1/15(6.6%)	3/27 (11.1 %)		
SCC		1/27 (3.7 %)		
AGC, neoplastic	1/15(6.6%)		2/5 (40.0 %)	
NELM	7/15(46.6%)	4/27 (14.8 %)	1/5 (20.0 %)	27/27(100.0%)
Unsatisfactory	1/15(6.6%)	1/27 (3.7 %)	1/5 (20.0 %)	

Table 15: Performance indicators of cytology

	Sensitivity	Specificity	PPV	NPV
LSIL	35.7%	92.9 %	55.5 %	85.4%
HSIL/SCC	69.2%	95.5 %	90.0 %	84.3%
Atypical glandular cells	50.0 %	98.5 %	66.6 %	97.0%

Follow up data was available in 21 out of 45 cases. The follow up period ranged from 3 months to 36 months. Mean follow up period was 11.2 months.

Out of 15 cases of CIN 1, follow up data was available in 10 cases. There was complete regression in 2 cases. There was persistence of CIN 1 in 3 cases. 3 cases and 2 cases progressed to CIN 2 and CIN 3 respectively.

Out of 15 cases of CIN 2, follow up data was available in 7 cases. Complete regression was observed in 2 cases, persistence of CIN 2 in 1 case and progression to CIN 3 in 4 cases.

Out of 15 cases of CIN 3, 4 cases had follow up information. It was observed that 1 case progressed to squamous cell carcinoma and 3 cases persisted as CIN 3.

The time(in months) taken for progression into a higher grade lesion was estimated to be 16 months using Kaplan Meir curve.

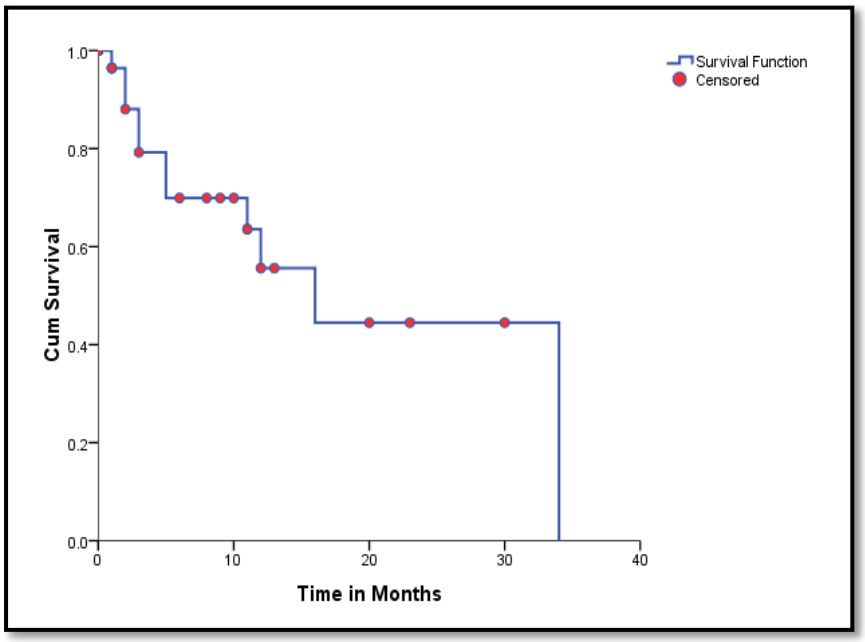

Figure 14: Kaplan Meir curve for assessment of progression into higher grade

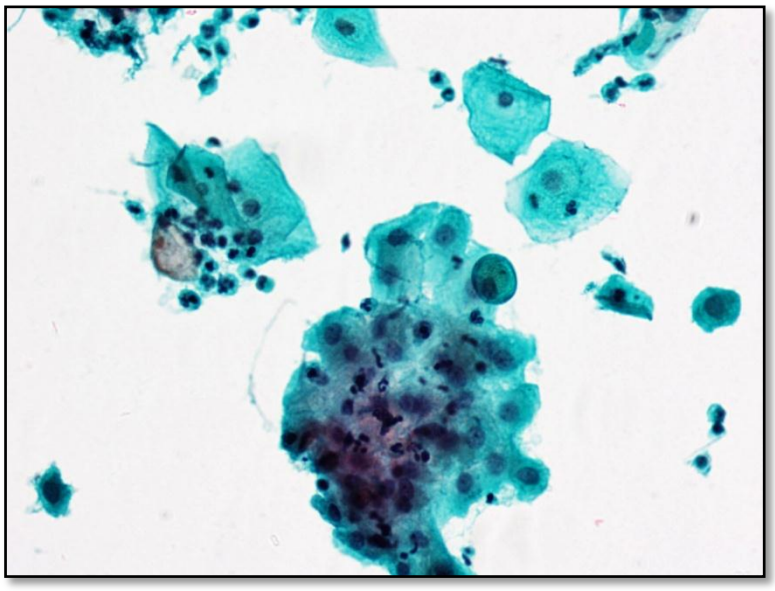

Figure 15: Thin Prep smear showing reparative change(20X)

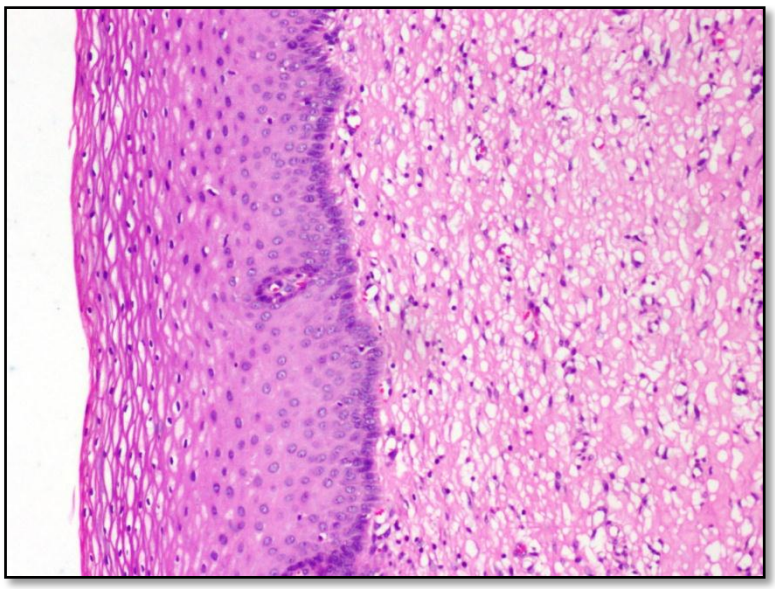

Figure 16: Stratified squamous epithelium of ectocervix with mild reparative change(H&E, 20X)

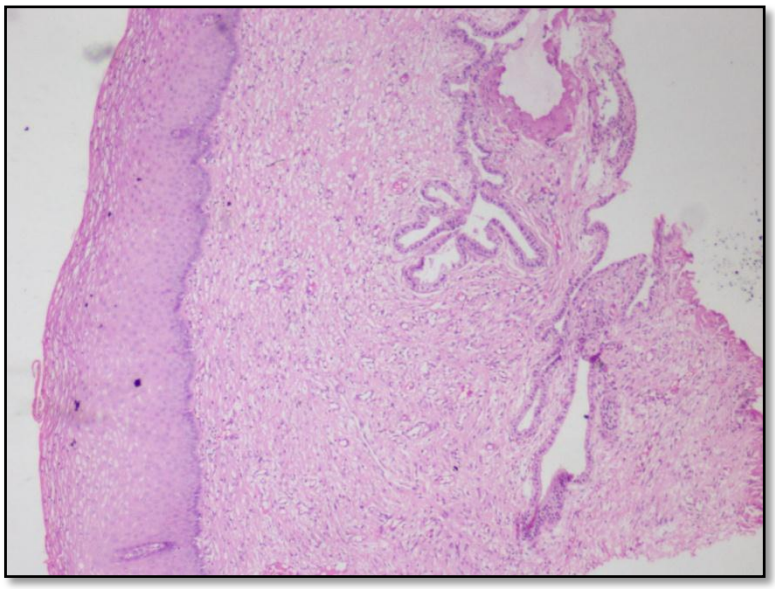

Figure 17: Stratified squamous epithelium with mild reparative change (H&E, 10X)

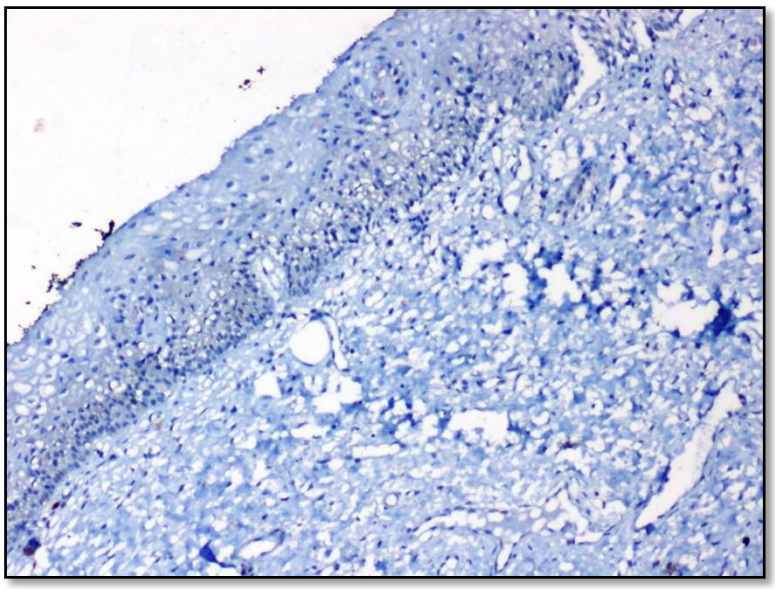

Figure 18: Absent p16 expression in reparative epithelium (10X)

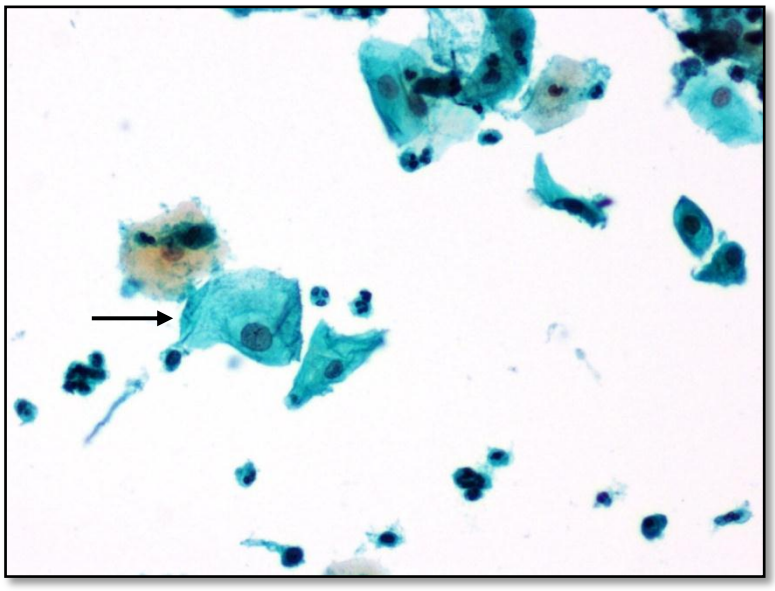

Figure 19: Thin Prep smear showing a koilocyte (20X)

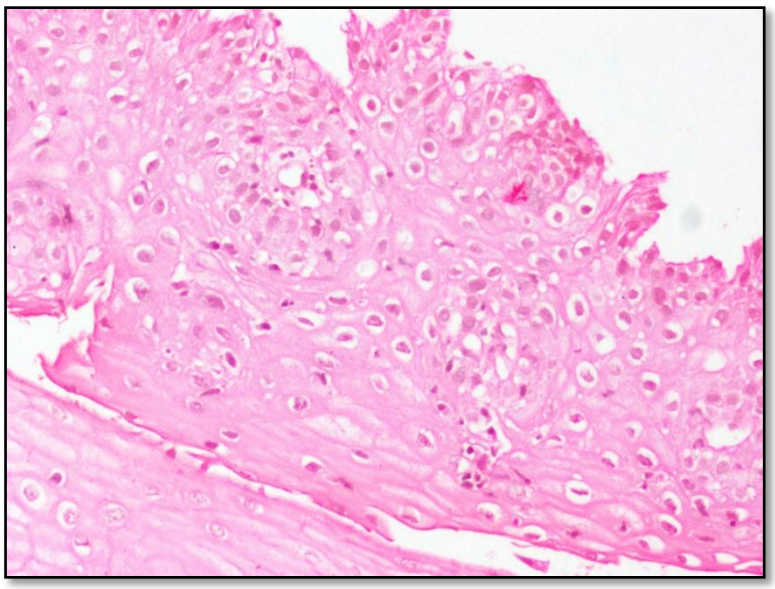

Figure 20: Ectocervical epithelium displaying koilocytosis (H&E,20X)

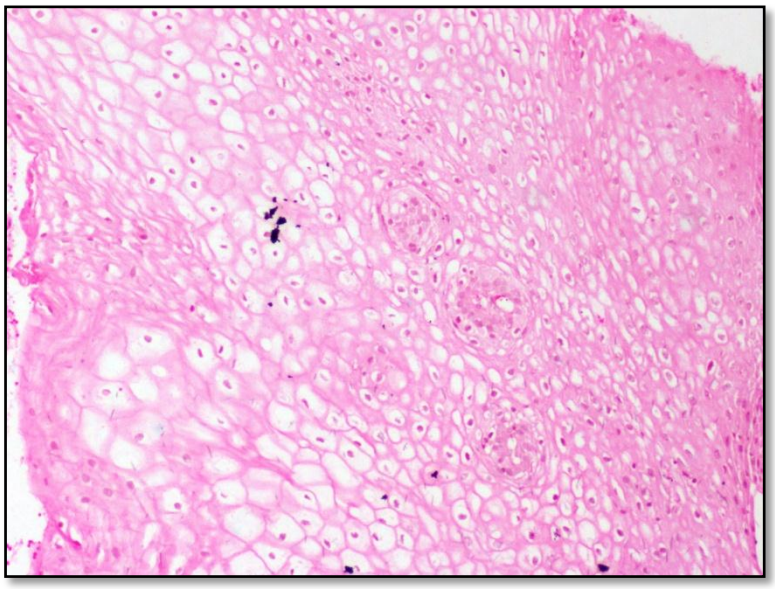

Figure 21: Ectocervical epithelium with koilcytosis (H&E,10X)

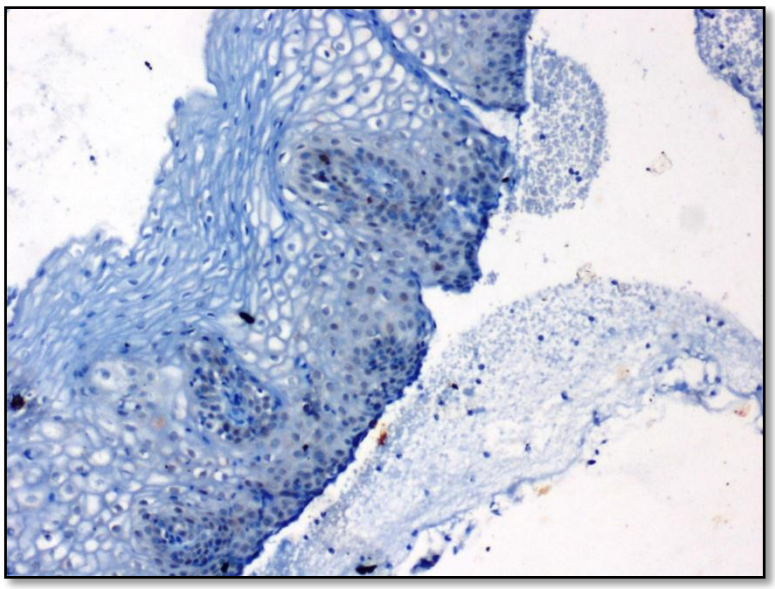

Figure22: Absent p16 expression in the koilocytes (10X)

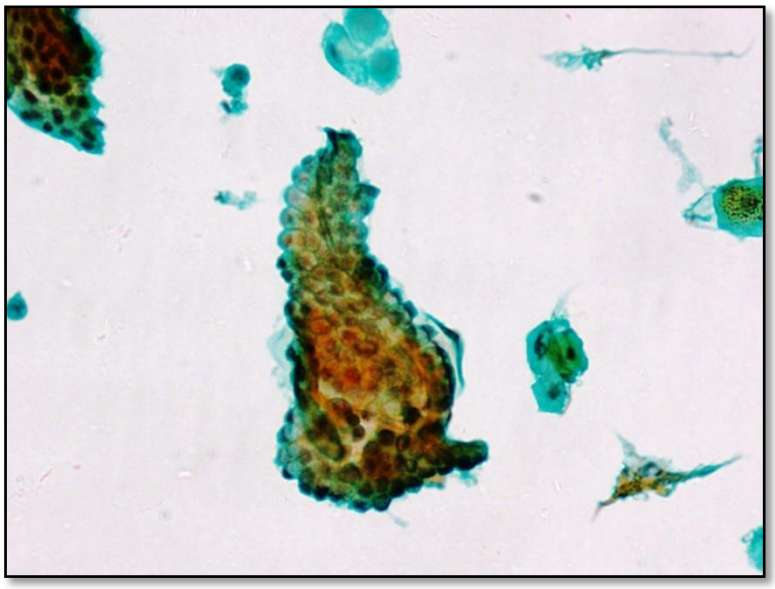

Figure 23: Thin Prep smears showing benign endocervical glands in microglandular hyperplasia (20X)

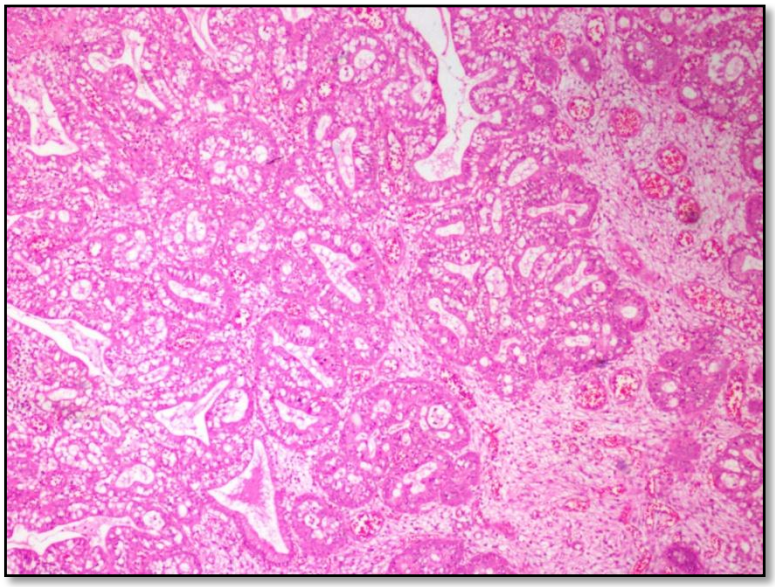

Figure 24: Microglandular hyperplasia (H&E,10X)

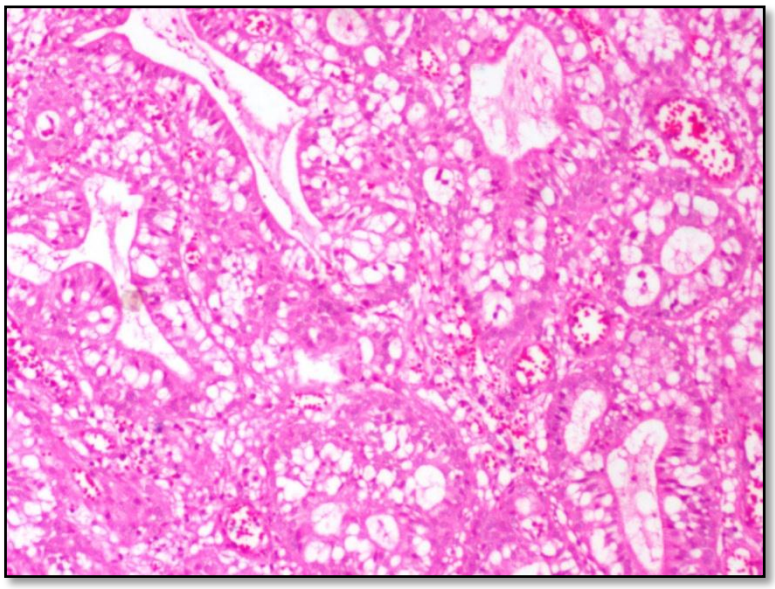

Figure 25: Microglandular hyperplasia(H&E,20X)

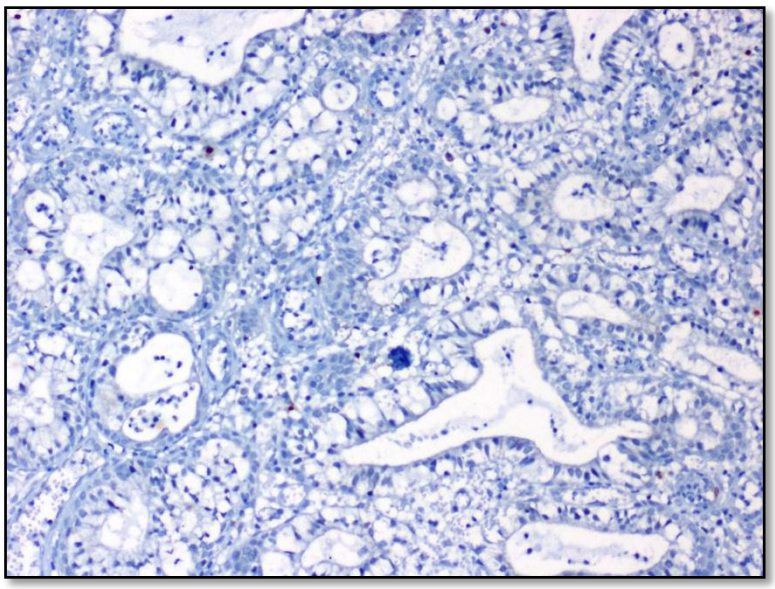

Figure 26: Absent p16 staining in benign endocervical glands(20X)

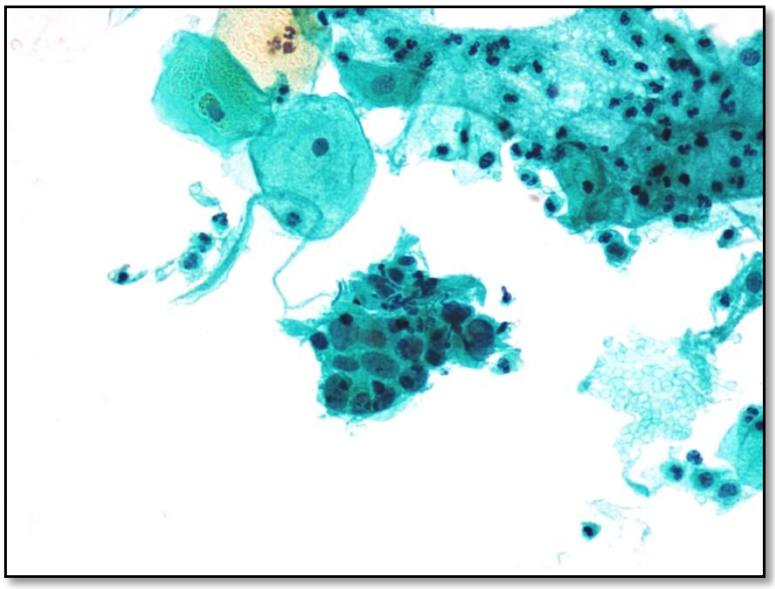

Figure 27: Thin Prep showing LSIL (20X)

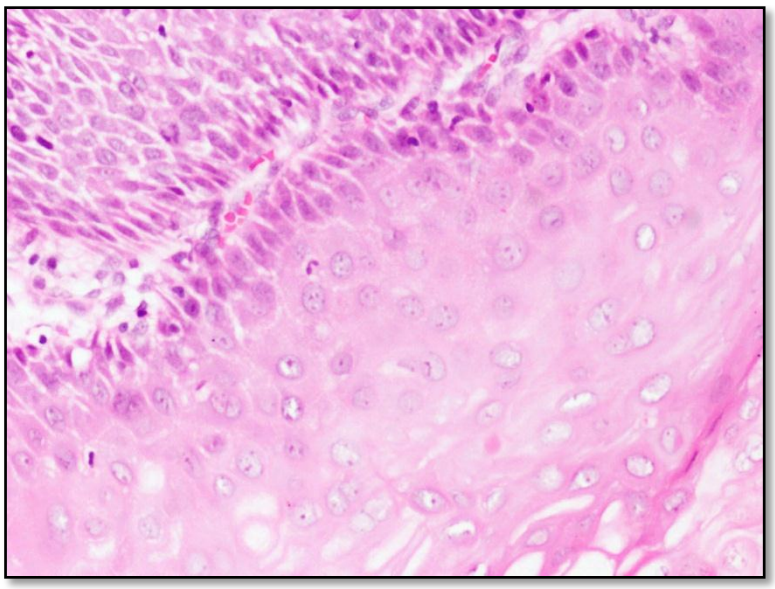

Figure 28: CIN 1 (H&E,40X)

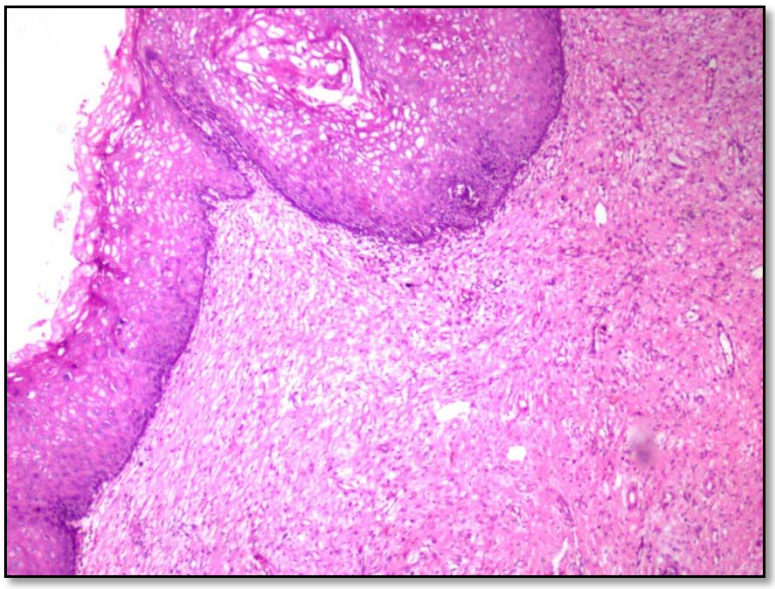

Figure 29: CIN 1 (10X)

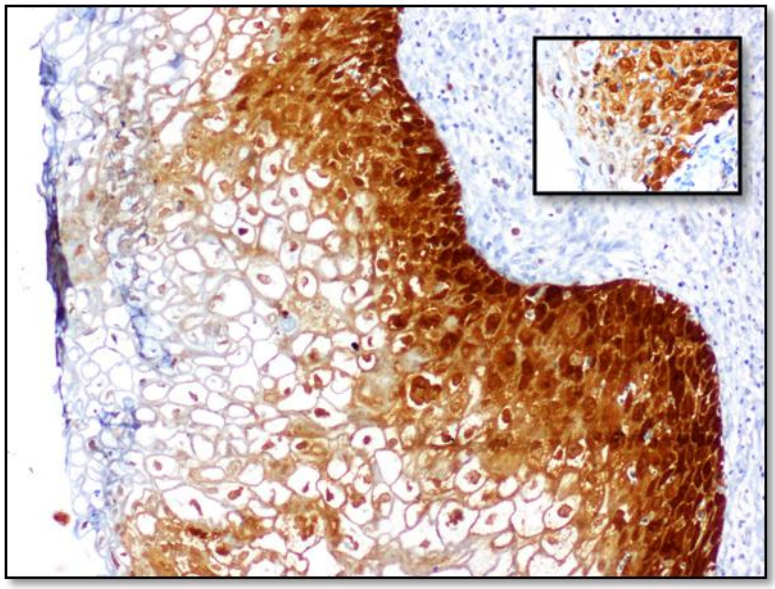

Figure 30: p16 staining the dysplastic epithelium in the lower one third (20X)

Inset: Nuclear staining by p16(40X).

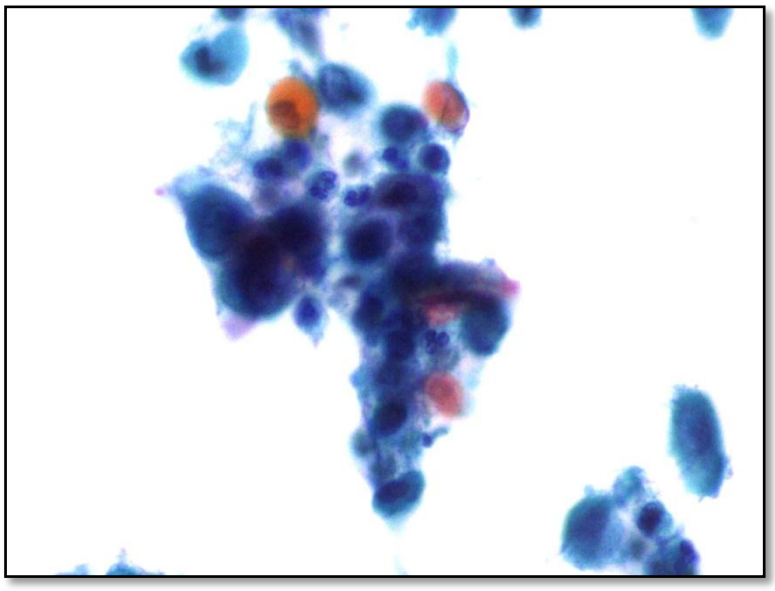

Figure 31: Thin Prep demonstrating HSIL (20X)

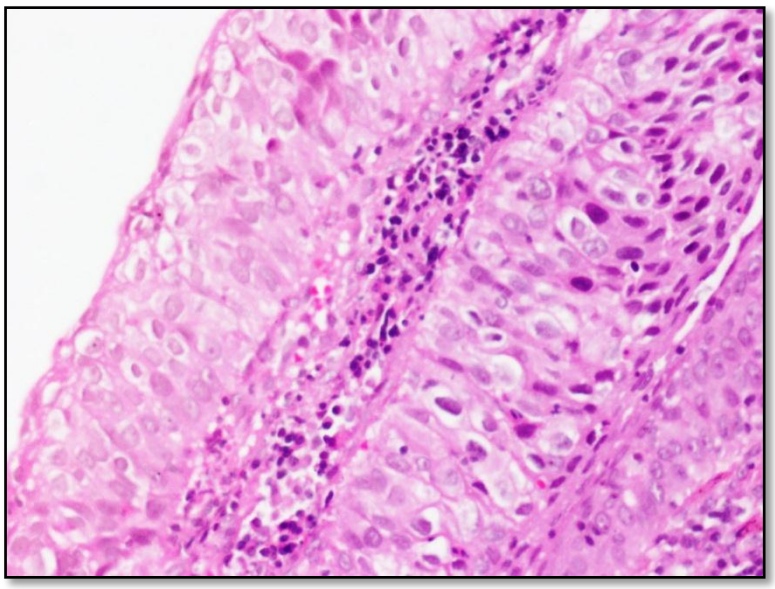

Figure 32: CIN 2 (H&E, 40X)

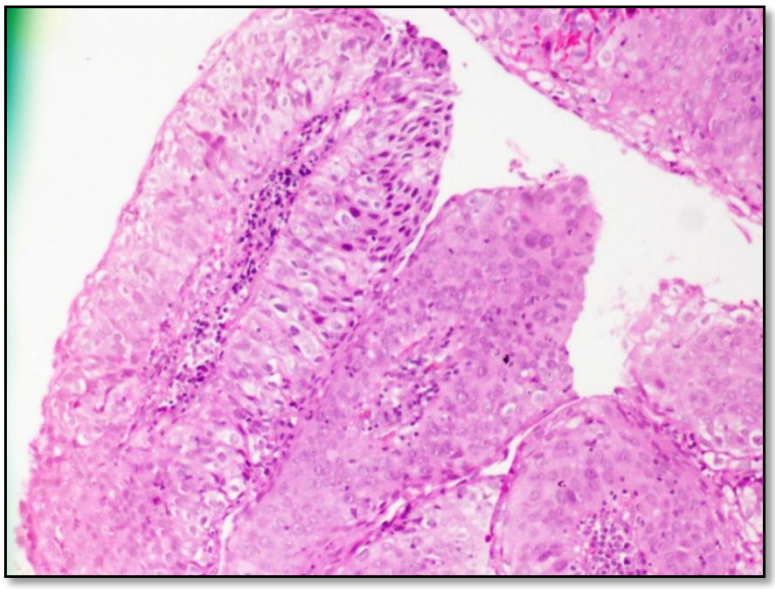

Figure 33: CIN 2 (10X)

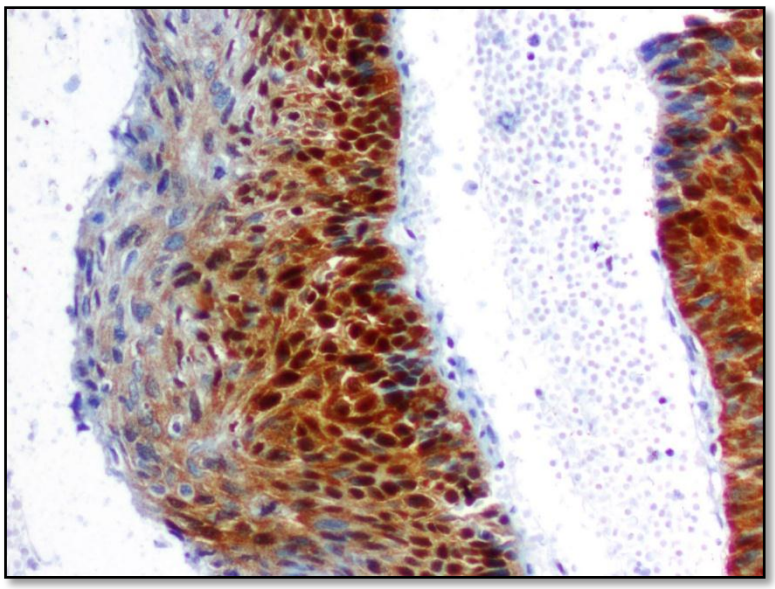

Figure 34: p16 staining the dysplastic epithelium in lower two thirds(20X)

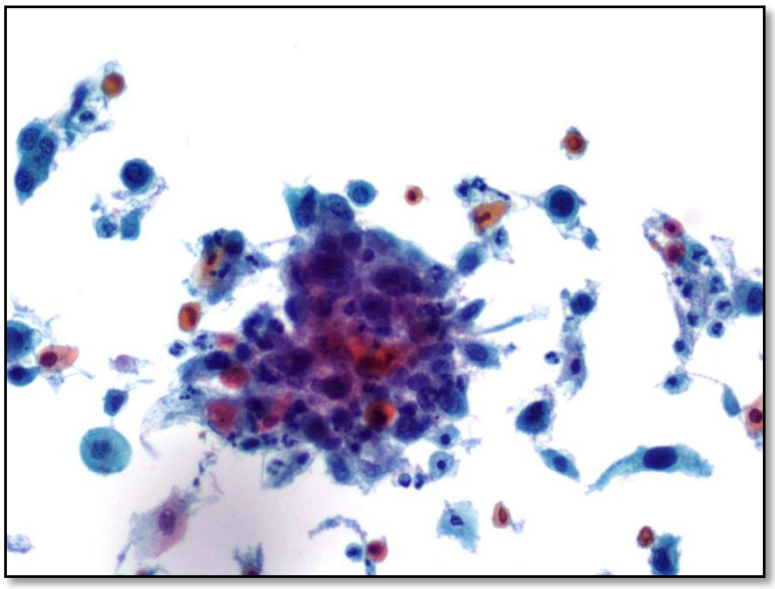

Figure 35: Thin Prep demonstrating HSIL (10X)

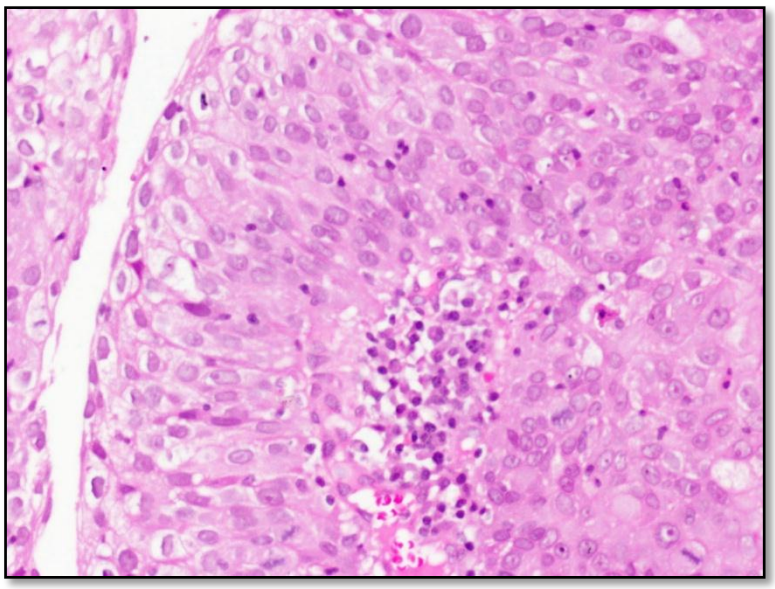

Figure 36: CIN 3 (H&E, 40X)

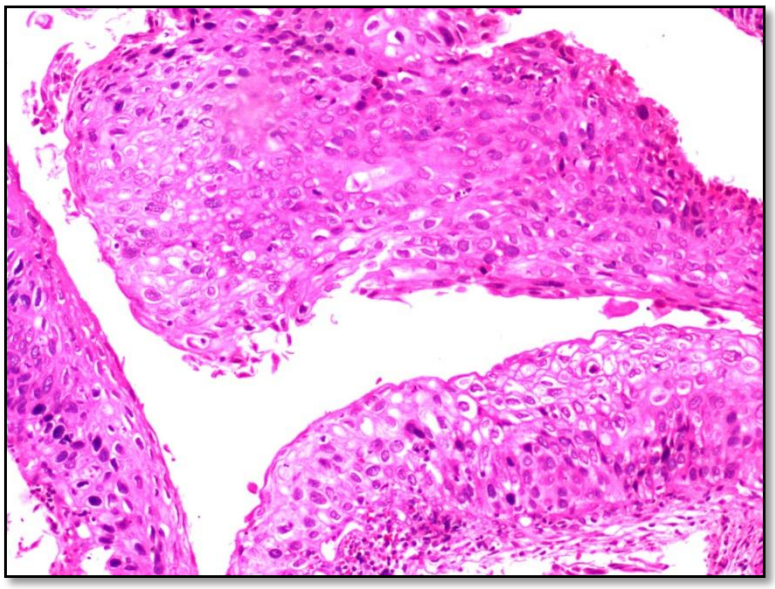

Figure 37: CIN 3 (H&E,20X)

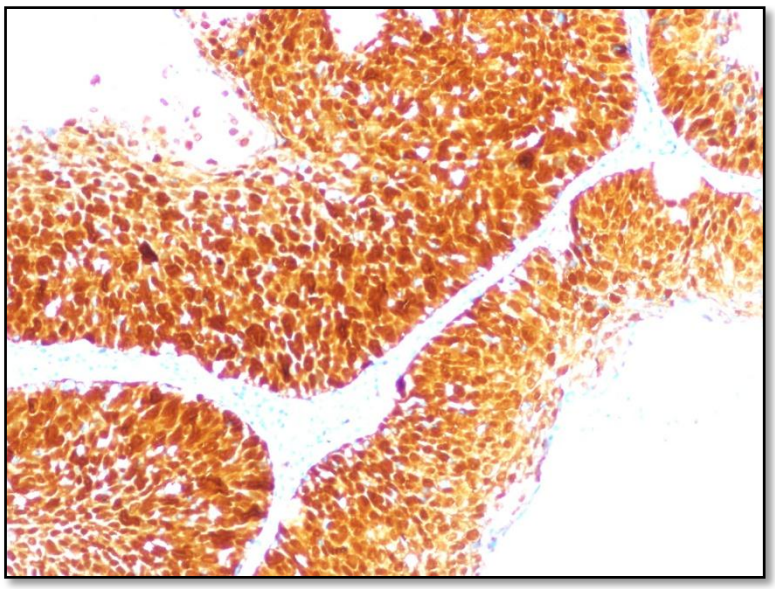

Figure 38: p16 staining the dysplastic epithelium full thickness(20X)

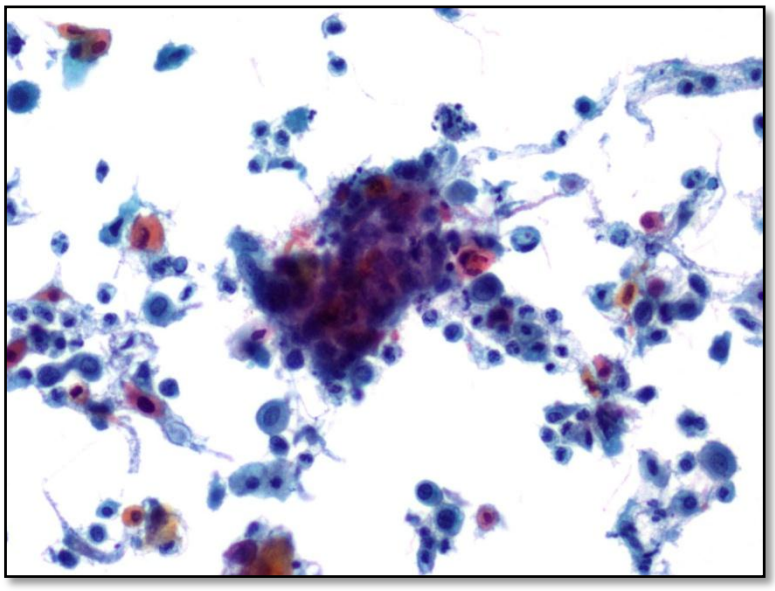

Figure 39: Thin Prep smear showing Squamous cell carcinoma(10X)

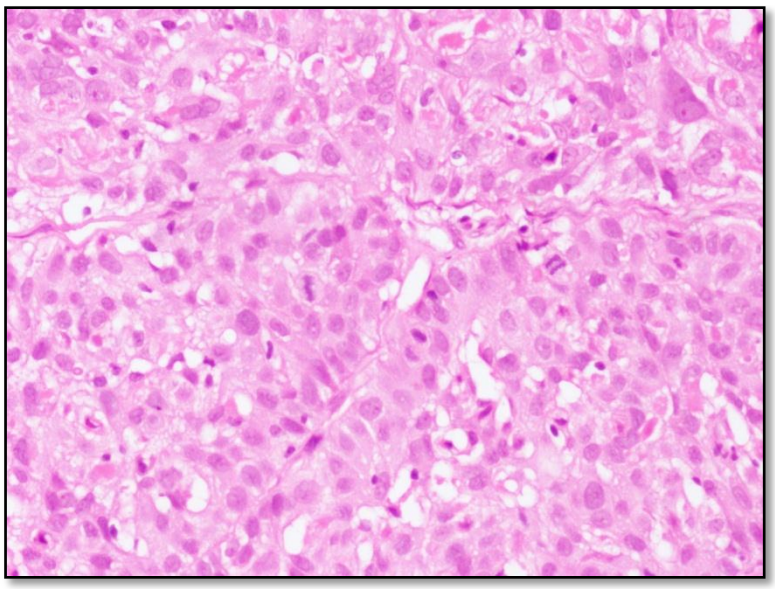

Figure 40: Squamous cell carcinoma (H&E,40X)

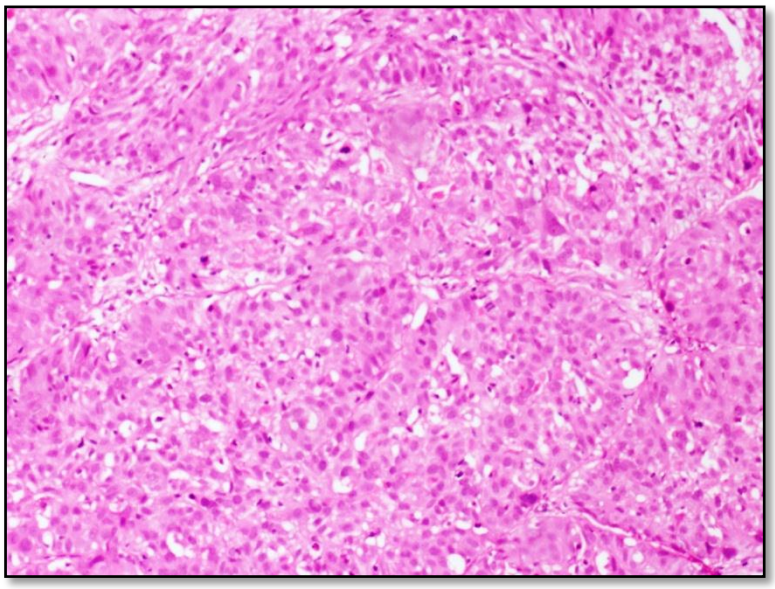

Figure 41: Squamous Cell Carcinoma (H&E, 20X)

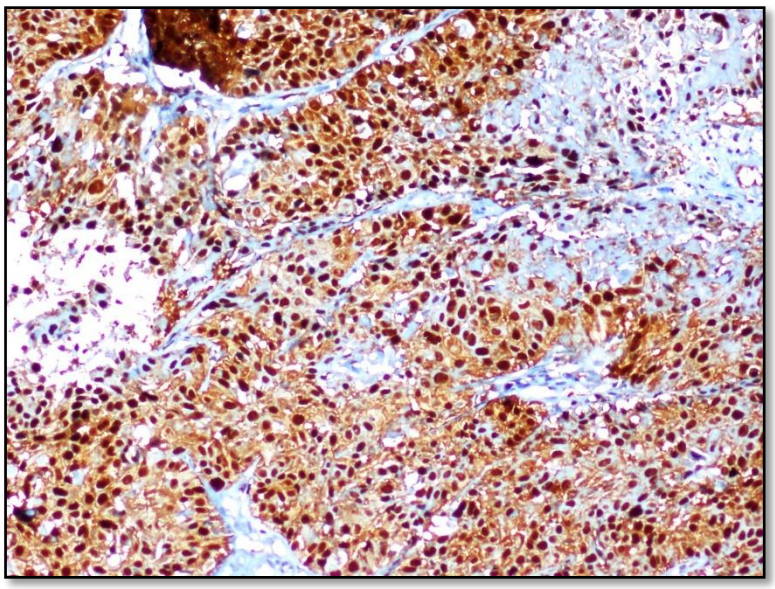

Figure 42: Diffuse and strong p16 staining in Squamous Cell carcinoma(20X)

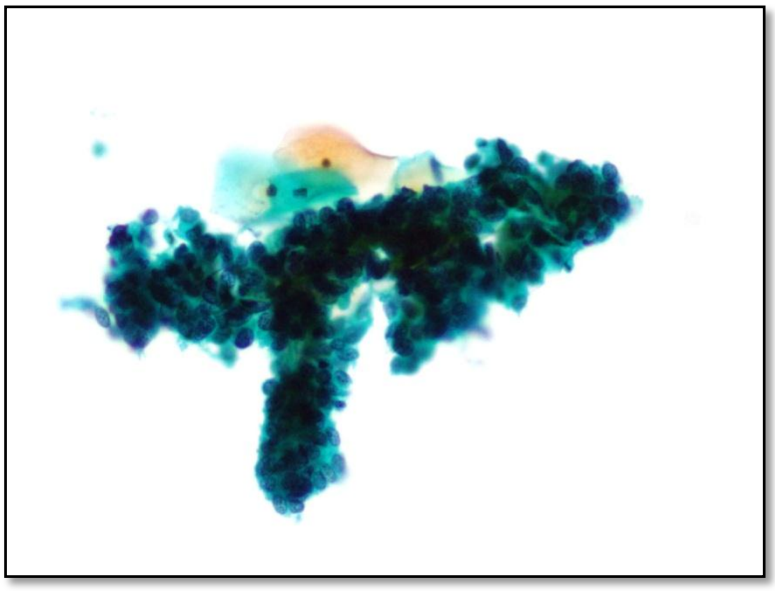

Figure 43: Atypical glandular cells, favour neoplastic (20X)

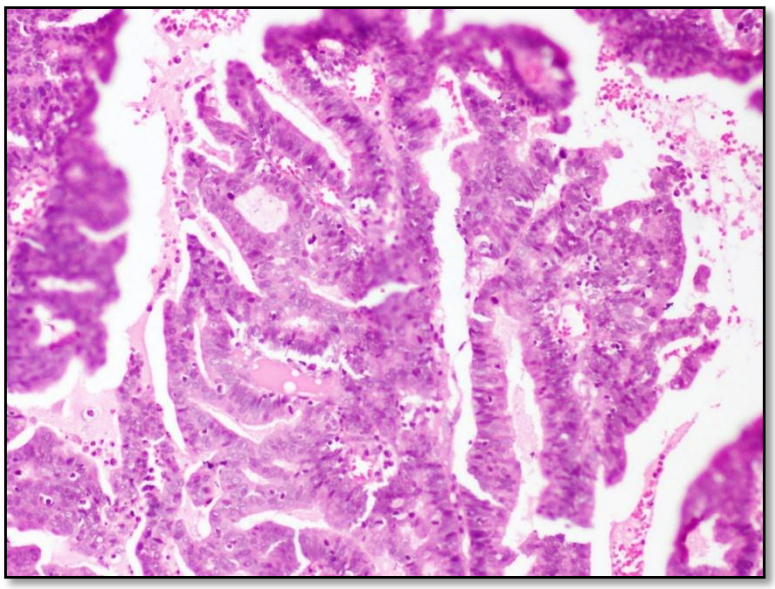

Figure 44: Adenocarcinoma(H&E, 40X)

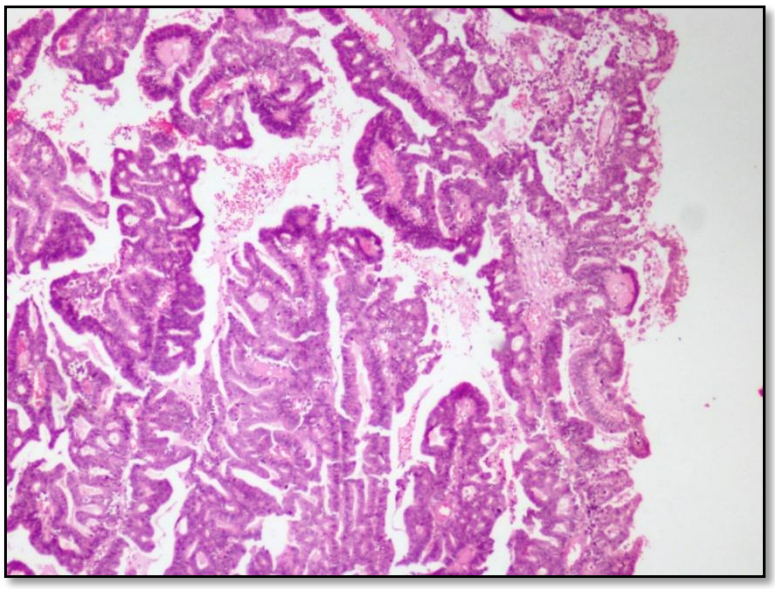

Figure 45: Adenocarcinoma (H&E,10X)

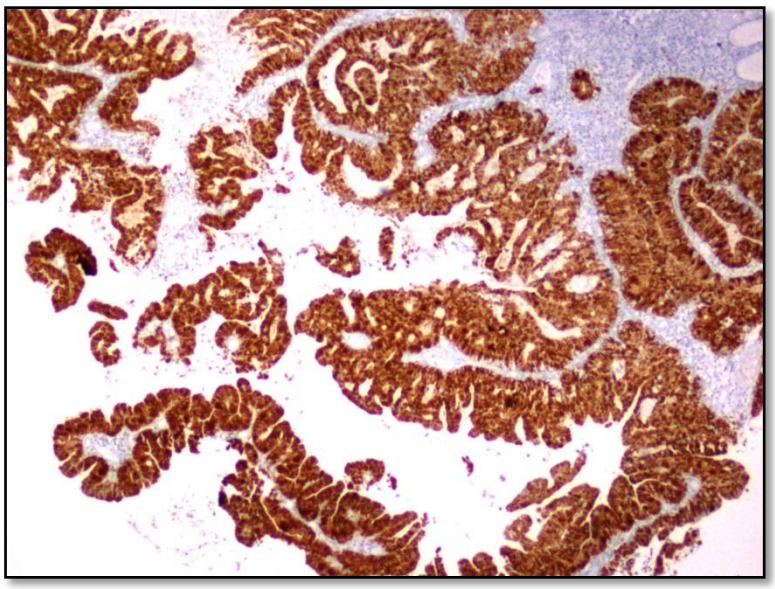

Figure 46: p16 staining in adenocarcinoma(10X)

DISCUSSION

India is a zone of high prevalence for cervical carcinomas with 20 % of new cases diagnosed every year(2). As cervical carcinomas evolve from distinct premalignant conditions, early detection and treatment plays a large role in reducing cancer related morbidity and mortality. Pap smear test, which is a simple and cost effective mass screening tool has drastically reduced the incidence of cervical cancer in developed countries over the past decades. As causal association of cervical neoplasia with Human Papilloma Virus was established, vaccines were developed thus, making it one of the few malignancies which are vaccine preventable.

The role of p16 and HPV has been extensively studied over the last two decades and has shown to play a major role in cervical tumourigenesis(58)(64). The biomarker, p16 has emerged as a reliable diagnostic as well as prognostic marker in cervical intraepithelial lesions. High risk HPV, most commonly subtypes 16 and 18 are responsible for almost all cases of cervical carcinomas. However, the mere presence of HPV in the cervical epithelium does not imply the presence of a neoplastic lesion. It is the persistence and integration of viral genome into the host that causes neoplastic transformation of epithelial cells(21). Hence, direct HPV DNA detection, though has a high sensitivity is not sufficiently specific to detect CIN or carcinoma.

Studies have shown that overall prevalence of high risk Human Papilloma Virus in inflammatory, CIN grades 1, 2 and 3 and invasive cancers to be 37.6%, 63.5%, 97.2% and 92% respectively. The most prevalent subtype was HPV16 (19). However, it was necessary to establish alternate methods other than HPV DNA detection due to high cost and a relatively low specificity.

Pap smear testing is universally accepted and widely used as a screening tool to detect premalignant and malignant conditions of cervix. Various studies have proven that Pap test has variable sensitivity and sufficiently high specificity to detect cervical dysplastic lesions(40)(37).

The aim of our study is to investigate the role of p16 as a surrogate marker for cervical dysplastic lesions and carcinoma and to evaluate the diagnostic accuracy of cervical cytology compared to corresponding cervical biopsy samples. A total of 120 cases were chosen with relatively more number of CIN cases (15 cases each of CIN 1, 2 and 3) as they are more diagnostically challenging than frank carcinomas. Considerable interobserver variation has been observed in interpreting both cytology and biopsy samples. Hence, an objective "gold standard" which would be HPV DNA detection should have been performed in all cases. But as the cost of this test would be high, we have chosen only the cases that have adhered strictly to the diagnostic criteria mentioned in Robboy's Pathology of Female Genital Tract to ensure accurate histomorphological diagnosis.

Clinical Features

In this study, majority of the patients with cervical carcinoma were from Tamil Nadu (54.05%). The highest incidence of cervical cancer in India is reported from Tamil Nadu (Age adjusted Incidence rate: 30.6 per 1, 00,000 women)(10). Among the 284 samples received, the incidence of squamous cell carcinoma was much higher than adenocarcinoma which is in concordance with the world demographics (85 % prevalence for squamous cell carcinoma compared to 15 %) for adenocarcinoma(90).

The mean age of women with squamous cell carcinoma and adenocarcinoma was found to be 52.0 years and 50.0 years respectively. Studies from Western literature observe

two peaks at 30-34 years and 80-84 years(90). The mean age of women with CIN 1, CIN 2 and CIN 3 were found to be 43.3 years, 41.4 years and 48.6 years respectively. A study done in United States (Kaiser Permanente health plan) observed highest rates of LSIL between 15 to 19 years of age and HSIL between 25 to 29 years of age(11) whereas; a study from India revealed mean age of LSIL as 32.3 years and HSIL as 40.5 years(33). This difference in the mean age of diagnosis could be due to the fact that more number of young women undergo routine cervical screening in developed countries thus enabling earlier detection of dysplastic lesion.

In the present study, the most common presenting symptom was vaginal discharge similar to the study done by Bal et al(33). Cervical growth was the most common presenting symptom in women with carcinoma. However, it is encouraging to note that 15.8 % of our patients had undergone cervical biopsy following routine Pap smear examination which detected cervical cytological abnormality thus implying that there is increasing awareness and access to screening programmes for women in our study cohort.

42.2 % of the patients had undergone LEEP/LLETZ procedures. Many of the patients with carcinoma presented with advanced stage of disease (Stages III and IV: 47.1% as against 38.8 % in Stage I and 13.8 % in Stage II). 35.1 % of them had nodal metastasis. 45.9% and 13.5% had pelvic and distant metastasis at the time of presentation respectively. This is in concordance with another Western study which states that stage of presentation is high in spite of effective screening programme(6).

Morphological features

Out of all the morphological features assessed (Nuclear hyperchromasia, enlargement, membrane irregularity, loss of polarity, mitotic count); it was observed that mitotic count was the only feature which showed statistically significant difference within the subtypes of CIN. A study done on cytological samples had shown the degree of nuclear membrane irregularity as the most significant differentiating feature(43). A study done by Aoyama et al on cervical biopsies states that is advisable to take into account all the morphological features before diagnosing cervical dysplastic lesions(44). We suggest that combination of all histological features with emphasis on mitotic count should be done to make a diagnosis of cervical dysplasia.

Immunohistochemical staining pattern of p16

In our study, we found that the sensitivity of p16 to detect intraepithelial lesions increase with increasing severity of the lesion (73.3% to 100% from CIN 1 to Squamous Cell Carcinoma). . Endocervical adenocarcinoma also showed a sensitivity of 95.4 % for p16 expression. The specificity of p16 to detect dysplasia was also sufficiently high (85.7 %). The positive predictive value ranged from 73.3 % to 78.9 % (CIN 1 to SCC) and was as high as 91.3 % in adenocarcinoma. The negative predictive values were 85.7 %, 100.0 % and 88.9 % in CIN, SCC and adenocarcinoma respectively. This is in keeping with the data available from similar cohorts worldwide(3)(62)(26). The study done in PGI, Chandigarh had got considerably lower sensitivity of p16 for CIN 1 (20%) but the findings were similar in high grade lesions (90% sensitive for squamous cell carcinoma)(71). The other Indian studies have similar expression profile(72)(73).

Table 16: Comparison of sensitivity of p16 obtained in other similar studies

	Present study	Lesnikova et al	Mood et al	Gupta et al
CIN 1	**73.3 %**	72.3 %	81.8 %	20.0 %
CIN 2	**93.3 %**	91.0 %	91.0 %	45.0 %
CIN 3	**93.3 %**	98.3 %	90.0 %	55.0 %
SCC	**100.0 %**	98.5 %	90.0 %	95.0 %
Adenocarcinoma	**95.4 %**		75.0 %	

p16 has been proven to have a very high discriminatory ability to distinguish between normal or reactive epithelium with dysplastic epithelium. Our study showed patchy weak membranous and cytoplasmic positivity in 6 reactive cases. A few other studies have observed this aberrant expression pattern as well(58)(62)(64). This is thought to be due to the presence of concomitant low grade HPV that produce cytological abnormalities too subtle to be detected on H&E sections. Moreover, none of the cases showed strong nuclear positivity.

Table 17: Comparison of proportion of normal cases with aberrant p16 expression in other similar studies

	Present study	Murphy et al	Tsoumpu et al	Mood et al
Normal / reactive cases with p16 expression	**15.7 %**	10.0 %	12.0 %	10.0 %

One case of moderately differentiated adenocarcinoma and 1 case of CIN 3 showed lack of staining and 1 case of poorly differentiated squamous cell carcinoma showed moderate staining for p16. This is due to differential expression of cell cycle regulator genes in HPV positive and HPV negative carcinomas. Studies have shown that HPV

positive cancers up regulate p16, cyclin E and cyclin B and down regulate cyclin D1 due negative feedback inhibition of Retinoblastoma activity as a result of E7 interaction. HPV negative cancers show reverse pattern of p16 and cyclin D1 expression. Reduced expression of p16 in HPV negative cancers are thought to be due to promoter hypermethylation and elevated cyclin D1 expresssion due to gene amplification(77).

It has been consistently observed in all the studies that low grade lesions have a reduced expression of p16. This can be explained by the fact that many of the low grade lesions are associated with low grade HPV in which E7 has a reduced affinity for Rb protein and failure of E6 to interact with p53(27). Low risk HPV are thought to interfere with Notch signalling pathway.

A diagnosis of frank carcinoma can be made even without an immunohistochemical marker. However, distinction of low grade intraepithelial lesion from marked regenerative or reparative change is diagnostically challenging usually requiring an immunohistochemical marker. The sensitivity of p16 to detect CIN 1 was found to be 73.3 % and 46.6 % with a score of 2 and 5 respectively. It is comparable to the results obtained in most of the other similar studies. CIN 1 and many cases of CIN 2 develop in latently infected epithelial cells caused by multiple subtypes of HPV. High grade lesions are usually associated with a single high risk subtype, usually HPV 16 which causes abortive infection in the epithelial cell and dysregulation of viral genome with higher number of E7 copies produced and thus promoting E7-Rb protein interaction.

Our study found a significant association between koilocytes and CIN 1 and CIN 2 but not with CIN 3. This is in keeping with other similar studies. Koilocytic atypia cannot be reliably distinguished with CIN 1 in many cases on H&E sections alone. It is controversial whether the presence of koilocytosis alone carries the adverse risk of

CIN1. It is therefore, essential to distinguish between the two lesions. All cases of koilocytosis were negative for p16 in the present study. Therefore, we propose that p16 can be used as a reliable marker to distinguish between CIN 1 and koilocytosis.

It was observed in this study that a small proportion of cases (6 out of 38) showed a patchy weak cytoplasmic / membranous positivity for p16 and another small proportion of neoplastic cells (1 case each of adenocarcinoma and CIN 3) showed absent staining. So we tried to evaluate the reliability of p16 in terms of intensity and proportion of expression. It was found that intensity was a better indicator of severity of lesion than proportion, in tune with another study which obtained similar results(3). Thus, a focal but strong and crisp expression is a more reliable indicator of the severity of lesion than a weak and patchy expression.

The prognostic significance of tumour grade is well known. Patients with well differentiated tumours are known to have a better overall survival than the patients with poorly differentiated tumour. We observed no statistically significant difference in the expression patterns of p16 in well, moderately or poorly differentiated tumours. (P value = 0.9). However, we got a statistically significant association between p16 expression and tumour stage, nodal status and metastasis. Tumours which over expressed p16 was associated with a more advance stage, positive lymph nodes and presence of metastasis (P value = 0.01, 0.01 and <0.01). The findings are consistent with a study done by Huang et al(6). As p16 is more strongly expressed in high grade lesions compared to low grade lesions, it is suggested that p16 may be involved in progression of lesion. More studies are required to elucidate the exact molecular mechanism.

Table 18: Comparison of prognostic value of p16 over expression with a similar study

	Present study	Huang et al
p16 over expression correlated with	**Stage of tumour, lymph node metastasis, pelvic and distant metastasis**	Stage of tumour, lymph node metastasis, pelvic and distant metastasis, histological subtype, overall survival
p16 over expression did not correlate with	**Grade of tumour**	Grade of tumour, lymphovascular invasion, parametrial and vaginal invasion, size and depth of tumour.

The cause for drastic reduction in the incidence of cervical carcinomas in developed countries has been attributed to effective implementation of screening programmes. A number of studies have been done both at national and international levels to assess the efficacy of cervical cytology screening programme. 15.8 % of patients with intraepithelial lesion were referred for biopsy due to abnormal cytology detected in Pap smears.

Our study showed LSIL (12 %,) HSIL (22 %) ASC H (5%), SCC (1%) and AGC (4%). The rate of ASC H was slightly higher compared to another similar Indian study done by Bal et al(33). The overall sensitivity of cytology was 58.9 % and specificity was 90 %. This is comparable to the data provided by national and international studies which showed a sensitivity ranging from 45% to 60 % and specificity 75 % to 90 %(29)(39)(35). We suggest that repeat evaluation of all cytology specimens by at least two cytopathologists can improve the sensitivity of Pap smears in detecting dysplasia. Other modalities which are coming up are image analysers and chemiluminescent speculoscopy.

Thus, in our institution the Pap testing has a sensitivity and specificity comparable to worldwide statistics.

Table 19: Comparison of sensitivity and specificity of Pap smears with other similar studies worldwide

	Present study (high grade lesions)	Nguyen et al	Denny et al	American Family Physician	Schechter et al	Renshaw et al
Sensitivity	**69.2 %**	85%	44-78%	55.4%	84.4%	50-75%
Specificity	**95.5 %**	90-99%	91-96%	94.1%	80.0%	

Table 20: Comparison of sensitivity and specificity of Pap smears with other similar studies in Asia

	Present study	Ansari et al (India)	Deodhar et al (India)	Moy et al (China)
Sensitivity	**69.2 %**	70-80 %	67.7 %	80.2 %
Specificity	**95.5 %**		95.4 %	93.3 %

It is well known that asymptomatic HPV infection is highly prevalent in sexually active women. However, only a small proportion of them develop an intraepithelial lesion.

60 % of LSIL and 30 % of HSIL are thought to regress, 30 % and 60 % of LSIL and HSIL persist and 10 % progress to a higher grade lesion within 2 years of initial diagnosis(27). Our study showed that 47.6 % of patients progressed to a high grade lesion, 33.3% persisted and 19.0 % of lesions regressed to a lower grade lesion. The follow up period ranged from 3 months to 36 months with a mean follow up of 11.2 months. The time taken for progression to a higher grade lesion was 16 months (Kaplan Meir curve). This warrants a close follow up of patients with Pap screening at least once a year in women with cervical epithelial abnormality.

Limitations of the study:

(1) The gold standard taken was cervical biopsy which is subject to interobserver variation. However, the slides were reviewed by two pathologists to reduce this.

(2) The diagnosis was not confirmed with HPV DNA detection by PCR due to financial constraints. We plan to obtain further funding to compare our results with PCR.

(3) Staining heterogeneity was noted, especially in cases of CIN 1. The intensity of staining varied within cases. This could be due to technical factors. Most of the test results with discrepancy were repeated to confirm.

(4) The sample size was small for a disease with high prevalence, again due to financial constraints.

(5) Clinical and follow up data were not available on all patients as this was a retrospective study and some of the patients were lost to follow up.

Recommendations

This study has accomplished the aims and most of the objectives that it targeted to achieve. However, there is a scope for further work in this field that can lead to early detection and treatment of cervical cancers thereby reducing the mortality rates.

Panel of markers:

Various studies have proved that use of a panel of markers considerably improves the sensitivity and specificity of diagnosis. The recommended panel would be p16, MIB-1, and cyclin D.

Distinction from endometrial adenocarcinoma:

As adenocarcinoma can arise from endometrium as well as cervical glandular epithelium, it is difficult to ascertain the origin of tumour morphologically. As p16 expression is HPV mediated and association of HPV with endocervical adenocarcinoma has been proven, p16 can be used to distinguish endometrial carcinoma from endocervical carcinoma. More studies are required in this field.

Incorporation of HPV DNA testing:

Since HPV DNA testing is considered the "gold standard", all women with cervical cytological abnormality should undergo HPV DNA testing to subtype into high risk and low risk categories, as women harbouring high risk HPV are more likely to progress into a higher grade lesion. The knowledge of prevalence of HPV infection will also enable the implementation of HPV vaccination strategy more effectively.

Genetic profiling of cervical tumours.

A better understanding of molecular basis of cervical tumours will help in diagnosis and prognostication. More studies are required in this field to predict the lesions that would undergo regression or progression into a higher grade.

Maintenance of hospital based registry to document the prevalence of premalignant and malignant lesions of cervix

As many of the high grade lesions are known to progress into an invasive carcinoma, women with cervical cytological abnormalities should be kept on close follow up. It is also important to know the prevalence and incidence of cervical carcinoma to implement effective vaccination strategy. Women should also be educated regarding the need for surveillance so that the number of patients lost to follow up can be reduced.

Improving the sensitivity of Pap smear testing

Pap smear test is still considered the most efficient screening tool in less developed countries. Active surveillance is required to detect early dysplastic lesions. We recommend that all cytology smears should be reviewed by two cytopathologists so as to increase the sensitivity of Pap smears.

CONCLUSION

- The sensitivity of p16 was 73.3 % in CIN 1, 93.3 % in CIN 2/3 and 100.0 % in squamous cell carcinoma. The sensitivity of p16 to detect endocervical adenocarcinoma was 95.4 %.
- The protein p16 is a reliable diagnostic marker to distinguish between various grades of dysplasia and malignancy.
- p16 can effectively distinguish benign mimics from dysplastic cervical lesions.
- Intensity of p16 expression correlated with severity of the lesion better than the proportion of staining.
- Over expression of p16 was associated with higher stage, nodal metastasis, pelvic and distant metastasis. p16 expression did not correlate with grade of tumour.
- Pap test had a low sensitivity to detect low grade lesions (35.7 %) whereas the sensitivity for high grade lesions was sufficiently high (69.2 %).
- Koilocytosis was significantly associated with CIN 1 and CIN 2 and not with CIN 3 lesions.
- Among the morphological features assessed to diagnose cervical dysplasia, mitotic count was the only parameter which showed significant difference among the subtypes of CIN and carcinoma.
- Most common presenting symptom in women with cervical dysplasia was vaginal discharge and women with carcinoma presented with cervical growth.
- 47.1 % of women with carcinoma presented with advanced stage (Stage III & IV).
- The time taken for progression of lesion to a higher grade was 16 months.

The results obtained in our study are in keeping with other studies conducted worldwide. We suggest that p16 immunohistochemical marker should be incorporated into routine cervical histopathological examination to improve the detection rate of premalignant lesions thus, enabling early detection and prevention of cervical carcinoma. However, more studies are required to unfold the molecular mechanisms of cervical carcinogenesis. The sensitivity of cervical cytology can be increased by concomitant use of p16 IHC at least in ambiguous cases. Thus, morphological, clinical and immunohistochemical parameters should be taken into account while diagnosing cervical dysplasia. Pap smear test as a screening modality would be reasonably effective as 15.8 % of asymptomatic women were detected to have cytological abnormality in our study. We also recommend that women with epithelial abnormality should undergo regular follow up at least once a year to prevent progression into higher grade lesions.

APPEENDIX 1

OFFICE OF RESEARCH
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Additional Vice Principal (Research)

February 21, 2014

Dr. Nivedita Suresh
PG Registrar
Department of General Pathology
Christian Medical College
Vellore 632 004

Sub: **Fluid Research grant project:**
P16 as a marker for cervical neoplasias: CIN, cGIN and invasive carcinomas in cervical biopsies.
Dr. Nivedita Suresh, PG Registrar, General Pathology, Dr. Ramani Manoj Kumar, General Pathology.

Ref: IRB Min. No. 8548 [OBSERVE] dated 12.11.2013

Dear Dr. Nivedita Suresh,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
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Cc: Dr. Ramani Manoj Kumar, General Pathology, CMC

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APPENDIX 2

Cervical dysplasias and carcinomas – A histomorphological and immunohistochemical study

PROFORMA

Serial No: Hospital No: Biopsy No:

Name: Age:

Clinical:

Mode of presentation:

Cervical cytology:

Radiological findings, if any:

Type of biopsy:

Gross:

Micro:

Nuclear abnormalities:

1) Increased nuclear cytoplasmic ratio: Present [ ] Absent [ ]

2) Hyperchromasia: Present [ ] Absent [ ]

3) Nuclear pleomorphism: Present [ ] Absent [ ]

4) Wrinkling of nuclear membrane: Present [ ] Absent [ ]

Mitotic activity:

1) Number of mitotic figures per10 high power fields:

2) Abnormal configurations: Present [ ] Absent [ ]

3) Loss of polarity in the epithelium:

Nil [ ] Basal one third [ ] Basal two thirds [ ] Full thickness [ ]

Inflammatory infiltrate: Conspicuous [ ] Inconspicuous [ ]

Viral koilocytic change: Present [ ] Absent [ ]

Glandular lesions:

1) Intracellular mucin: Present [ ] Absent [ ]

2) Increased nuclear cytoplasmic ratio: Present [ ] Absent [ ]

3) Hyperchromasia: Present [ ] Absent [ ]

4) Nuclear pleomorphism: Present [ ] Absent [ ]

In case of carcinoma:

Extent of invasion, if present: <3mm [ ] >3mm [ ]

Solid areas, if present (in case of glandular lesions):

<5% [ ] 5-50% [ ] >50% [ ]

Adjacent cervical tissue - Dysplasia Present [ ] Absent [ ]

Lymphovascular invasion: Present [ ] Absent [ ]

Impression:

Category:

IHC:: Intensity (0/1+/2+/3+) Proportion(0-10%, 10-25% 25-75% and 76-100%) and pattern (C/M/N)

Antibody	**Tumour focus**			**Adjacent Cervical Tissue**	**Control**
P 16	Intensity	Proportion	Pattern		

Other immunohistochemical markers done:

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